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# EXPERIMENTAL CELL RESEARCH

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# A METHOD OF FIXATION FOR ENZYME-CYTOCHEMISTRY AND CYTOLOGY

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Cytochemical methods for demonstrating enzymatic activities within cells are often applied to material which has been fixed in chilled acetone or alcohol. Acetone is usually preferred when different enzymes are to be demonstrated in the same material, since alcohol apparently destroys the acid phosphatase. The techniques suffer from a serious disadvantage, because both fixatives, and especially acetone, are unsatisfactory for the preservation of cytological details.

The work presented below reports on a good cytological fixative which does not markedly affect the commonly looked for enzymatic activities.

## MATERIALS AND METHODS

For most experiments rat organs were used. Pieces of brain, liver, kidney and small-intestine were put into various fixing solutions at different temperatures within five minutes of death. In some experiments pieces of organs from human autopsy material were used within 6 to 8 hours after death. The pieces were less than 2 mm in thickness and up to 1 cm in diameter.

Fixation lasted for 4 hours and was followed by dehydration overnight in absolute alcohol or acetone at room temperature. Control slices were fixed in chilled acetone together with every set of experiments. Clearing in two changes of benzene, 20 minutes each, was followed by embedding at 56° in three changes of paraffin, 10 to 15 minutes each, at a reduced pressure (vacuum of about 600 mm Hg).

Consecutive sections were stained with hematoxylin and eosin, and for five different enzymes. 1) Alkaline phosphatase, according to Gomori's technique (1), 2) Acid phosphatase, according to the recently improved technique of Gomori (2), 3) Lipase, according to Gomori (1), using Tween 40, 4) Phosphamidase, according to Gomori (3), 5) Cholinesterase, according to Gomori (4), using myristoyl choline as substrate.

Sections were kept in the substrate solutions overnight. No counterstaining was done.

## EXPERIMENTS AND RESULTS

Fixation at +4° Centigrade with various dilutions of alcohol and acetone in water did not improve the cytological picture, nor were the enzymes

better preserved than in the undiluted fixatives. Carnoy's fluid was unsatisfactory as most enzymatic activities were markedly reduced.

Isopropanol, chloroform and dioxane were not found to be satisfactory. The addition of acetic acid 5–10 per cent to all the fixing agents greatly improved the cytological results. Enzymes other than acid phosphatase were not destroyed by the addition of the acid, provided the fixing solution contained no water.

In other experiments the effect of chilling was studied. It was found that the same fixatives gave unsatisfactory cytological appearances when fixation was done at temperatures ranging between –15° and approximately –40°. There was marked evidence of ice-crystal formation, with consequent distortion and laceration of cells. On the other hand, it was found that enzymatic activities were better preserved for most fixatives than at higher temperatures.

An attempt was made to find a temperature range within which both cytological structure and enzymatic activities would be well preserved. Our experiments have shown that with acetic acid 5 per cent in absolute alcohol at temperatures below zero, the degree of preservation of enzymatic activities (including that of acid phosphatase) increased with the decrease in temperature. The same effect was noted with regard to the quality of the cytological fixation. The optimal temperature appeared to lie somewhere in the neighbourhood of –8°, at which no ice crystals were found. This method of fixation, i.e. 5 per cent acetic acid in absolute alcohol, at –8° to –6° C, for 4 hours, followed by dehydration with absolute alcohol at room temperature gave us excellent results: cytological fixation was good; enzymatic activities, including that of acid phosphatase, were actually better preserved than in control slices which were fixed in chilled acetone.

The temperature of –8° to –6° can be easily obtained in any laboratory. The fixing mixture is put into the freezing compartment of any electrical refrigerator where such range of temperatures can be easily produced.

## SUMMARY

1. A method for fixation of tissues which allows good preservation of cellular structures and at the same time causes less inhibition of the five enzymes tested than chilled acetone, is described.

2. The method consists of fixation of thin slices of tissue in an absolute alcohol-acetic acid mixture (19 +1 vol. s) at a temperature of –8° to –6°

for four hours, followed by dehydration in absolute alcohol at room temperature overnight.

3. Excellent results were obtained by this method with rat and human material, including brain, liver, intestine and kidney tissues, both in regard to cytology and to enzymatic activities of alkaline and acid phosphatases, lipase, phosphamidase and cholinesterase.

The authors are greatly indebted to Dr. G. Gomori of the University of Chicago, for his kindness in supplying them with samples of myristoylcholine and p-Cl-anilidophosphonic acid.

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# DIE QUANTITATIVE AUSWERTUNG DES HISTIOTOPEN ESTERASENNACHWEISES

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Eingegangen am 22. September 1951

Zum histiotopen Nachweis von Enzymen in Geweben und Organen stehen uns heute zahlreiche Verfahren zur Verfügung. Übersichtsreferate finden sich bei Gomori (6), Glick (4) und Bradfield (1), technische Angaben besonders bei Glick (5). Alle diese Verfahren bergen aber zahlreiche Fehlerquellen in sich und sollten nur unter kritischer Berücksichtigung aller Möglichkeiten, die zu falschen Resultaten führen könten, verwendet werden.

In einer früheren Arbeit (13) untersuchten wir die zahlreichen Fehlerquellen des zuerst von Gomori (7, 8) angegebenen Verfahrens zum Nachweis von Esterasen *in situ*. Aus unseren Messungen und Beobachtungen ergaben sich verschiedene Abänderungen der ursprünglichen Technik, die wesentlich zur Verbesserung der Methode in Bezug auf Spezifität, Aktivitätsverluste während der Schnittherstellung, Sensibilität und Konstanz der Reaktion beitrugen. In einer späteren Arbeit (14) machten wir Angaben über die Verteilung einiger Organesterasen bei der Albinomaus. An gleicher Stelle findet sich auch eine Übersicht über die unseres Wissens publizierten Arbeiten mit dem histiotopen Esterasennachweis.

Methoden wie die unsrige und die meisten anderen histiotopen Nachweisreaktionen sind zwar geeignet, die Lokalisation bestimmter Stoffe innerhalb eines Organes nachzuweisen, besitzen aber im Gegensatz zu den gebräuchlichen physikalisch-chemischen Verfahren den Nachteil, dass sich aus ihren Resultaten keinerlei quantitative Schlüsse ziehen lassen. Für experimentelle Untersuchungen sind heute aber rein qualitative Aussagen meist ungenügend. „Obviously, it would be preferable to establish both the quantitation and the topographical disposition of these biologically significant components" (4).

Da wir auf Grund histochemischer Untersuchungen wissen, dass in bestimmten Organen, wie zum Beispiel in der Schweineleber (Abb. 1, 2, 3), die Verteilung der Esterasen gleichmässig ist, oder mit anderen Worten, dass alle Parenchymzellen annähernd gleich viel Esterase enthalten, können wir

die Aktivität der einzelnen Zelle ohne weiteres durch Division der physikalisch-chemisch bestimmten Aktivität durch die Zellenzahl in derselben Masseinheit Organsubstanz berechnen. Nun zeigen aber die wenigsten Organe eine gleichmässige Verteilung, sondern die Enzyme liegen lokalisiert in bestimmten Regionen und Zellen. So findet man bereits in der Mausleber (Abb. 4) eine Verteilung, die eine Bestimmung der Enzymaktivität der einzelnen Zellen im oben angegebenen Sinne unmöglich macht. Die Esterase liegt in diesem Organ nämlich ganz besonders im Zentrum der Leberläppchen, eng um die *Vena centralis* herum, während die Peripherie der Läppchen recht arm an Enzym sein kann.

Die ersten Versuche einer sowohl quantitativen als auch qualitativen Enzymbestimmung in Organen mit geschichtetem Bau, das heisst mit nicht gleichmässiger Enzymverteilung, stammen unseres Wissens von Willstätter und seinen Mitarbeitern (17, 18). Diese Autoren trennten makroskopisch die einzelnen Schichten des Schweinemagens und bestimmten titrimetrisch die Enzymaktivität derselben. Über die zellgetreue Lokalisation konnten aber mit dieser Methode keinerlei Aussagen gemacht werden.

Bedeutend genauere Angaben über die quantitative und qualitative Verteilung der Enzyme gestatten die Methoden, die von der Carlsberger Schule entwickelt wurden. Die besonders von Linderstrøm-Lang und Holter seit 1930 ausgearbeiteten Mikromethoden erlauben die Titration kleinster Säuremengen in Mikrotomschnitten. Abwechselnd wurden in schichtförmig gebauten Organen (Magen-Darmtraktus) einerseits oberflächenparallele Schnitte mikrochemisch auf ihren Enzymgehalt geprüft, anderseits wurde an histologischen Präparaten eine Zählung der verschiedenen Zelltypen durchgeführt. In der Annahme, dass ein bestimmtes Enzym an einen bestimmten Zelltyp gebunden sei, wurde dann durch statistischen Vergleich versucht, eine Gesetzmässigkeit zu finden, die erlaubt, die Enzyme bestimmten Zelltypen zuzuschreiben. Referate über die allgemeinen Grundlagen dieser Technik finden sich, ausser in den schon zu Beginn angeführten Arbeiten, bei Holter und Linderstrøm-Lang (11) und Linderstrøm-Lang und Holter (12). Die spezielle Technik zur Bestimmung der lipolytischen Aktivität wurde von Glick (2, 3) ausgearbeitet. Den unfixierten Gefrierschnitten wurde als Substrat Methylbutyrat zugeführt und die entstehende Buttersäure azidimetrisch bestimmt. Dieses Verfahren ist aber nicht nur äusserst umständlich, sondern es birgt auch zahlreiche Fehlerquellen in sich. Aus diesen Gründen schien es vorteilhaft, eine präzisere und zugleich einfachere Methode zu suchen.

Ausgehend von seiner histochemischen Nachweisreaktion hat neuerdings Gomori (10) ein Verfahren zur quantitativen Auswertung seiner Reaktion

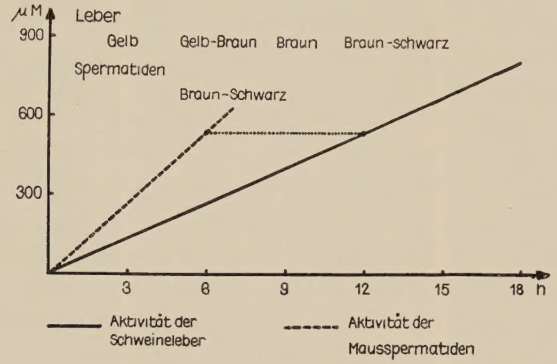
angegeben. Zu diesem Zweck wurden Organhomogenat-Ausstriche von bekannter Enzymaktivität auf Objektträgern denselben Manipulationen unterworfen wie die Gewebeschnitte beim Esterasennachweis. Die Enzymschicht von bekannter Aktivität und Dicke zeigte nach einer bestimmten Zeit eine gewisse Reaktion, deren Intensität mit derjenigen der Mikrotomschnitte unbekannter Aktivität verglichen wurde. Wir haben schon vor der Publikation Gomori's ein ähnliches Verfahren versucht, dessen einziger Unterschied darin bestand, dass wir die Menge des Enzympräparates von bekannter Aktivität nicht wie Gomori durch Wägung, sondern durch Ausmessung bestimmten, indem wir die Dicke einer gleichmässigen Enzym-Kollodiumschicht direkt unter dem Mikroskop massen. Es zeigte sich aber, dass die so erhaltenen Resultate nicht stimmen konnten, indem der Enzymausstrich mit zunehmender Inkubationszeit eine relativ abnehmende Intensität im Vergleich zum nicht entparaffinierten Organschnitt zeigte. Wir erklären dies dadurch, dass die Diffusion im Enzympräparat bedeutend grösser ist als im Schnitt. Wir konnten früher schon zeigen (13), dass die Diffusion in nicht entparaffinierten Schnitten viel geringer ist, als in solchen, die mit einem Kollodiumfilm bedeckt sind. Ebenso zeigten wir dort, dass im Kollodiumpräparat die Reaktion gegenüber nicht entparaffinierten Schnitten erheblich verzögert ist. Diese beiden Fehlerquellen bewogen uns, von diesem Verfahren abzusehen und eine andere Bestimmungsmethode zu suchen.

## DURCHFÜHRUNG DER QUANTITATIVEN ESTERASENBESTIMMUNG

Wir bestimmten zuerst nach den schon früher gemachten Angaben (13) potentiometrisch die enzymatische Aktivität einer Schweineleber gegenüber Tween 60. Da der Spaltungsverlauf zu Beginn der Reaktion linear verläuft, genügte eine einzige Titration. Gleichzeitig entnahmen wir von derselben Stelle ein Stück Organ zur histochemischen Verarbeitung. Die Mikrotomschnitte wurden genau nach den früher gemachten Angaben hergestellt und während 3, 6 und 12 Stunden inkubiert. Nach Ablauf dieser Zeitspanne wurden die Schnitte entwickelt und entparaffiniert. Auf diese Weise bekamen wir in der zunehmenden Intensität der histochemischen Reaktion ein Abbild des chemisch bestimmten Spaltungsverlaufes (Tab. I) im Sinne einer kolorimetrischen Reihe (Abb. 1, 2, 3). Tab. I zeigt den Verlauf der Spaltung von Tween 60 durch ein Schweineleberhomogenat während 18 Stunden. Über der Kurve sind die Farbtöne der während gleichen Zeiten inkubierten Leberschnitte angegeben (obere Reihe). Wenn wir nur 4 Farbtöne angeben, so liegt das in der Beschränktheit unserer Ausdrucksweise. In Wirklichkeit lassen

TABELLE I.

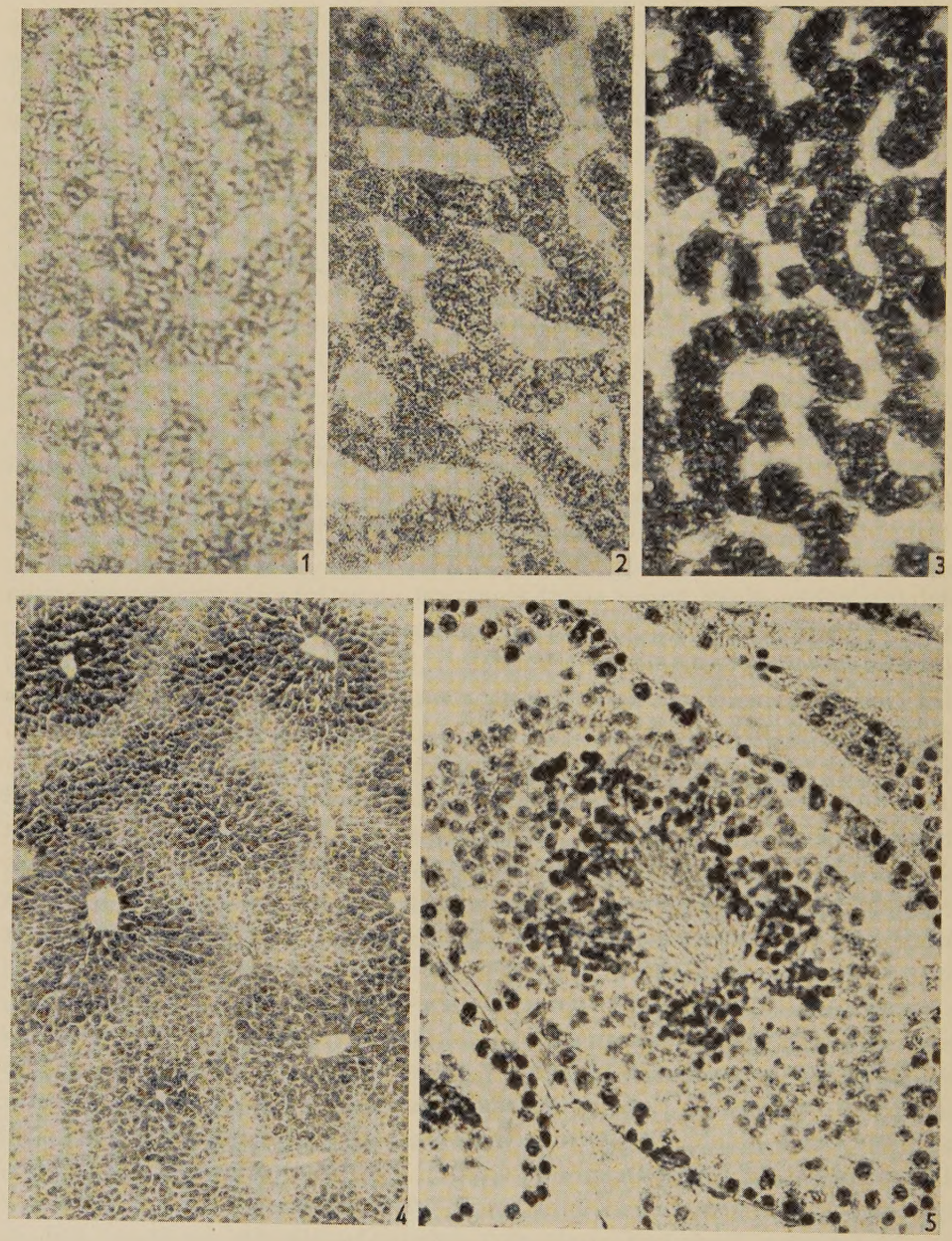
Die elektropotentiometrisch bestimmte Aktivität dieser Schweineleber betrug 50 μM Tween-60-Spaltung. Die Intensität der histochemischen Reaktion derselben Leber war nach 12 Stunden dieselbe, wie die der Spermatiden der Maus nach 6 Stunden. Das Schema zeigt den Verlauf der Spaltung durch die Spermatiden, die demnach eine esterolytische Aktivität von 100 μM aufweisen.




sich zwischen einer kaum sichtbaren und einer intensiv braun-schwarzen Reaktion zahlreiche Vergleichswerte herauslesen und es lässt sich so eine kolorimetrische Vergleichsskala aufstellen. Zeigen nun Präparate, die gleich, das heisst in derselben Serie, im gleichen Inkubationsgemisch und gleich lang inkubiert, behandelt wurden, eine entsprechende Intensität der Reaktion, so liegt der Schluss nahe, dass ursprünglich dieselbe Enzymaktivität vorlag. Da in der chemisch bestimmten Spaltungskurve die Aktivitätsverluste während der Herstellung der Schnitte nicht inbegriffen sind, so haben wir damit direkt eine Angabe über die ursprüngliche Aktivität des Materials.

Nach dem Vorschlage von Gomori (9) geben wir die Enzymaktivität in Mikromol (μM) der Spaltprodukte nach einer Reaktionsdauer von einer Stunde, bei Verwendung von 1 g Frischmaterial an.

Zeigt nun ein anderes Präparat aus derselben Serie nach einer kürzeren, bzw. längeren Inkubation dieselbe Reaktionsintensität, wie ein bestimmtes Präparat der oben beschriebenen Vergleichsreihe, so können wir daraus schliessen, dass seine Enzymaktivität grösser, bzw. kleiner sein muss. Wie sich in einem solchen Falle der Spaltungsverlauf konstruieren lässt, zeigt Tab. I. Ein bestimmter Braunton, einfach als „braun-schwarz" bezeichnet, erschien bei einem Leberschnitt nach 12 Stunden Inkubation (Abb. 3). Ein in derselben Serie behandelter Schnitt aus dem Hoden der Maus (Abb. 4) zeigte in den Spermatidenkernen dieselbe Intensität nach 6 Stunden (untere Reihe). Diese Spermatidenkerne spalten somit in 6 Stunden gleich viel




Schweineleber, Esterasereaktion nach eigener Modifikation (13), Inkubationszeit 3, 6 und 12 Stunden. Vergrösserung 300-fach.

Tween 60 wie die Leberzellen in 12 Stunden und damit können wir die neue Spaltungskurve durch den entsprechenden Punkt ziehen. Da nun die Leber, von der die hier besprochenen Präparate stammen, eine Enzymaktivität von 50 μM aufwies, können wir aus der Kurve entnehmen, das die Spaltung durch die Spermatidenkerne 100 μM beträgt.

## DIE FEHLERBREITE DIESER AUSWERTUNG

Gomori (10) gibt für seine oben skizzierte Methode eine Fehlerbreite von 50 % an. Wir möchten auch für unser Verfahren eine ähnliche Fehlerbreite annehmen, wenigstens was die Aktivitätsbestimmung esterasearmer Organe anbelangt. Für esterasereiche Schnitte nimmt die relative Fehlerbreite zweifellos ab. Die Fehlerbreite ist einmal allgemein von der Genauigkeit der chemischen Aktivitätsbestimmung abhängig. Dazu kommen noch spezielle Faktoren, wie der nicht exakt mögliche Farbvergleich, die nicht absolute Konstanz der Reaktion, die individuellen Schwankungen des Esterasegehaltes der einzelnen Zellen u.a. Wie Glick (5) hervorhebt, sind alle kolorimetrischen Verfahren verdächtig, da eine Reaktion nie genau gleich reproduzierbar ist. Es ist ja bekannt, dass selbst relativ einfache histologische Färbungen bei genauer Einhaltung derselben Vorschrift etwas differente Resultate geben; mit zunehmender Kompliziertheit der Reaktion potenziert sich dieser Fehler. Wir glauben ihn dadurch einigermassen umgehen zu können, dass wir für jede Präparatenserie immer gleichzeitig eine chemische und histochemische Kontrollbestimmung mit Schweineleber durchführen.

Gomori (10) macht auf eine weitere Fehlerquelle solcher kolorimetrischer Vergleiche aufmerksam. Er weist darauf hin, dass intensive Reaktionen auf kleinem Raum unterbewertet, diffuse Reaktionen eher überbewertet werden

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Abb. 1 zeigt eine noch schwache, diffuse, im Präparat leicht gelbe Reaktion.

Abb. 2 entspricht der braungelben Färbung nach 6-stündiger Inkubation. Die Esterase ist an feine, über die ganze Zelle verteilte punktförmige Ablagerungen gebunden. Die Kerne erscheinen stellenweise wie ausgespart. Ebenso sind die Zellgrenzen gelegentlich negativ.

Auf Abb. 3 ist die Reaktion so intensiv braun-schwarz, dass sich keinerlei Einzelheiten mehr in Bezug auf die genauere Lokalisation erkennen lassen. Hingegen erscheinen die Zellgrenzen auch hier gelegentlich als ausgesparte, weisse, esterase-negative Linien.

Aus dieser Bildreihe geht der Vorteil der sukzessiven Schnittentnahme deutlich hervor.

Abb. 4. Mausleber, Esterasereaktion nach eigener Modifikation, Inkubationszeit 6 Stunden. Leichte Kernfärbung mit saurem Hämalaun nach *Mayer* (16).

Die Esterase ist vorwiegend an die Umgebung der *Vena centralis* gebunden. Vergrösserung 50-fach.

Abb. 5 Hoden der Maus, Esterasereaktion nach eigener Modifikation, Inkubation 3 Stunden. Nur die Kerne der Spermatiden zeigen eine positive Esterasereaktion. Die Kerne der übrigen Zellen wurden nach der Reaktion mit saurem Hämalaun n. *Mayer* gefärbt. Vergrösserung 300-fach.

und dass ferner, sobald die Reaktion ihr Maximum erreicht hat, eine quantitative Auswertung unmöglich wird. Diese Fehlerquelle spielt bei unserem Verfahren kaum eine Rolle, denn durch die sukzessive Schnittentnahme nach 3, 6 und 12 Stunden lässt sich der Mittelwert dieser drei Bestimmungen ausrechnen, der von diesem Fehler nicht mehr stark belastet ist. Ebenso werden bei der sukzessiven Schnittentnahme Präparate dabei sein, bei denen die Reaktion noch nicht maximal ist.

Damit steht der quantitativen Aktivitätsbestimmung der lipolytischen Enzyme *in situ* nichts mehr im Wege und wir werden in einer späteren Arbeit (15) die Leistungsfähigkeit dieser Methode am Beispiel der Verteilung der Esterasen im Magen-Darmtraktus des Schweines zeigen. Das hier angegebene Verfahren dürfte sich bei entsprechender Modifizierung auch für andere histiotope Enzymreaktionen eignen.

## ZUSAMMENFASSUNG

Es wird eine Methode beschrieben, welche eine Schätzung der Lipaseaktivität einzelner Zellen und Zellsysteme erlaubt. Das Prinzip des Verfahrens besteht darin, dass die Intensität der histochemischen Esterasereaktion in Mikrotomschnitten unbekannter Aktivität mit Kontrollschnitten aus einem Organ mit bekannter Enzymaktivität verglichen wird.

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# LE PROBLÈME DE L'ULTRASTRUCTURE DU CYTOPLASME ET DES ARTÉFACTS DE FIXATION

## ÉTUDE SUR LES LEUCOCYTES ET LES PLAQUETTES SANGUINES AU MICROSCOPE ÉLECTRONIQUE

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Reçu le 3 Octobre 1951

Si, dans le domaine de la microscopie optique, on a appris à connaître les artéfacts de fixation, il est loin d'en être ainsi en microscopie électronique.

Ce n'est pas seulement parce que le pouvoir séparateur, cent fois supérieur à celui du microscope ordinaire, rend visibles des artéfacts imperceptibles jusqu'ici, c'est surtout parce que le microscope électronique nous donne accès à un monde dont les caractères structuraux, déterminés par l'état colloïdal, sont d'une «fluidité» infiniment plus grande que ceux des organites cellulaires bien définis.

La fixation la plus parfaite entraîne donc des modifications de l'ultrastructure. Il s'agit de les connaître. Mais là, nous nous heurtons à des difficultés presqu'insurmontables. Il faudrait pouvoir saisir les stades intermédiaires des transformations qui mènent de l'état vital à l'état fixé, comme on a pu le faire depuis longtemps en microscopie optique (voir p. ex. (19)), mais l'extrême ténuité des transformations qui nous intéressent ici et l'impossibilité d'observer une cellule vivante au microscope électronique nous privent de tout moyen de contrôle. De plus, les modifications que peuvent subir des tissus même fixés de façon parfaite, lors des manœuvres ultérieures (déshydratation — inclusion) se surajoutent et deviennent très apparentes même dans les cas où elles étaient négligeables en microscopie optique. Par contre, l'altération de la préparation dans l'instrument même (vide et faisceau électronique) ne joue guère de rôle, contrairement à ce que l'on pensait au début, à condition de ne pas pousser l'intensité du faisceau électronique au delà de certaines limites.

Ce problème fondamental, la conservation de la structure fine de la cellule, plusieurs travailleurs déjà ont cherché à le résoudre à l'échelle du microscope

électronique, en se basant sur les travaux classiques de la microscopie optique.

Citons Porter, Claude et Fullam, Bessis et Bricka, Fauré-Fremiet, Bessis et Thaureaux, Bairati et surtout Bretschneider à qui l'on doit les recherches les plus étendues sur les liquides fixateurs.[1]

Notre propre contribution expérimentale nous a amenés à nous intéresser plus particulièrement aux questions suivantes:

1. Existe-t-il une ultrastructure réelle du hyaloplasme et comment est-elle influencée par les fixateurs? Comment peuvent-ils faire disparaître ou au contraire exagérer une structure préexistante?

2. Cette structure fine du hyaloplasme représente-t-elle une architecture générale et valable pour des souches cellulaires différentes, ou bien existe-t-il une ultramorphologie spécifique pour une race cellulaire donnée?

3. Quel est le rôle des forces purement mécaniques (tension de surface, phénomènes d'étalement) dans l'orientation de cette structure?

4. Dans quelle mesure la microscopie électronique peut-elle apporter des faits morphologiques précis à l'appui des théories sur l'ultrastructure du cytoplasme, en particulier de la théorie des «points de jonction» (Haftpunkt Theorie) de Frey-Wyssling?

## MATÉRIEL ET MÉTHODE

Comme Bessis et Bricka, nous avons choisi pour notre expérimentation les leucocytes et les thrombocytes du sang humain normal. Ce matériel nous a paru particulièrement choisi pour cette étude parce qu'on obtient très facilement un grand nombre de cellules provenant de lignées sanguines différentes, bien étalées les unes à côté des autres sur la même préparation: des polynucléaires avant tout, quelques monocytes et beaucoup de plaquettes. Les lymphocytes ont été exclus de cette étude parce qu'ils ne s'étalent que rarement sur la membrane plastique (Formvar).

Les leucocytes s'étalent rapidement sur des lamelles enduites de Formvar qui se trouvent dans un tube contenant du sang frais à 37°. Après 20 minutes, la préparation est rincée légèrement au Tyrode et fixée 5′ ou 30′ dans le fixateur choisi. Après lavage et séchage, métallisation à l'or et examen au microscope électronique Trüb-Täuber 50 KV. (Pour tous les détails, voir nos publications antérieures.)

Nous avons ainsi utilisé environ une trentaine de fixateurs ou mélanges fixateurs classiques:

— *L'acide osmique à 2 % (vapeurs) et les mélanges à base d'acide osmiques:* Flemming, Benda, Meves, Champy.

[1] Depuis la réduction de ce travail, d'autres études sur ce même sujet ont paru, voir entre autres: Bairati, A., et Lehmann, F. E., *Revue Suisse Zool.*, **54**, No. 19 (1951); Kawamura, H., *Arch. histol. Jap.*, **2**, No. 2 (1951); Palade, G. E., *J. Exptl. Med.*, **95**, No. 3 (1952); Policard, A., Bessis, M., et Bricka, M., *Bull. Microscop. Appl.*, **2**, Nos. 3 et 4 (1952).

— *Le formol et les mélanges à base de formol:* Formol dilué: 1/4, 1/10, dilué au sérum physiologique 1/10. Bouin, Duboscq-Brasil.
— *Le bichromate de potassium et les mélanges à base de bichromate de potassium:* Tellyesnicsky, Kaformacetat, Regaud, Müller, Orth.
— *Le sublimé et les mélanges a base de sublimé:* Schaudinn, Lang, Heidenhain, Zenker, Helly, Maximow.
— *Les alcools:* Alcool éthylique, Mélange de Carnoy ou Van Gehuchten, Alcool méthylique.
— *D'autres substances:* L'acide chromique 1 % — l'acide picrique — l'acide phosphomolybdique 1 % — l'acide phosphotungstique à 1 % — le sulfate d'uranium — le chlorure de platine. Et différents mélanges de ces quatre derniers composés.

Etant donné qu'il s'agit toujours d'une couche monocellulaire, la vitesse de diffusion des différentes substances ne joue aucun rôle et la *fixation se fait immédiatement.* Nous n'avons pas vu de différence entre les préparations fixées 5′ ou 30′. Toutes les opérations ont été faites à la température normale du laboratoire.

En ce qui concerne l'importance du *facteur pH*, nous n'avons pas fait des recherches détaillées; si nous avons pu vérifier qu'une certaine acidité était nécessaire, les pH basiques entraînant la destruction de la cellule, et que le degré d'acidité de l'acide chromique et du bichromate de potasse revêtait une importance particulière nos images sont, de ce point de vue, plutôt contradictoires et ne nous ont pas permis de tirer de conclusions.

Nous avons admis comme condition minima d'une *fixation acceptable*, la parfaite conservation des contours cellulaires et l'absence de plages détruites au sein du cytoplasme.

La mise en évidence du chondriome et de la granulation spécifique ainsi que la conservation du noyau, n'ont été que des problèmes d'importance secondaire dans nos recherches.

## RÉSULTATS

Dans la majorité des cas, quel que soit le liquide utilisé, on retrouve des *fibrilles ou des réseaux hyaloplasmiques*; que cette structure fibrillaire ou réticulaire soit évidente ou au contraire qu'il faille la rechercher après une étude attentive, elle est presque constante. Plus rarement cependant l'image est plutôt d'apparence *granulaire.*

En gros, deux ordres d'aspects peuvent se rencontrer qui nous permettent de classer les fixateurs en *deux grands groupes*:

*Un premier groupe* où la trame hyaloplasmique est fine, régulière, homogène, quelquefois même, à peine apparente ou complètement effacée. Dans ces cas, les cellules ont un aspect finement grenu. Les fixateurs tels que l'acide osmique, le formol, le sublimé, appartiennent à ce groupe (Fig. 1 a, Fig. 2 a, b, c, Fig. 3 a, b, Fig. 4).

*Un deuxième groupe* où la trame est plus épaisse «grossière», réalisant

des aspects spongieux et alvéolaires. L'acide chromique, le bichromate de potasse, l'acide picrique, l'alcool, en sont les principaux représentants (Fig. 1 b, Fig. 2 d e, f, Fig. 3 c, d, Fig. 5 et 6).

Les images obtenues après fixation avec les substances de base indiquées se caractérisent en détail de la façon suivante:

a) *Le premier groupe:*

*L'acide osmique* (Fig. 1 a, 3 a, 4). Le protoplasme est *fin*, partout semblable, *homogène*, *compact*. En certaines zones, une trame granuleuse fine se dispose de façon particulière et prend une orientation, les éléments constituants se groupant en fines fibrilles, parallèles les unes aux autres. Les mitochondries, les enclaves lipidiques par contre se détachent avec une netteté particulière sur cette trame légère.

*Le formol* non dilué (Fig. 3 b). Comme pour l'acide osmique, l'aspect est *fin*, *homogène*, et *compact*, mais les unités élémentaires granuleuses paraissent plus souvent volumineuses et moins régulières. D'autres laissent voir un *réseau* dont les éléments constituants s'ordonnent en fibrilles courtes et fines. Ces aspects réticulaires deviennent évidents, «grossiers», sur les éléments qui paraissent moins bien fixés (formol dilué à 25 % et à 10 %, formol à *pH basique*). Il faut signaler un aspect particulier à ce fixateur: on trouve disséminées sans ordre au sein du protoplasme, des formations «cratériformes» limitées par un anneau en relief, le centre étant représenté par la trame de fond altérée.

*Le sublimé* (Fig. 2 a). Contrairement à toute attente, a sur le protoplasme un effet comparable et les trois mêmes qualificatifs peuvent le caractériser: la trame est *fine*, *homogène* et *compacte*.

b) *Le deuxième groupe:*

*Le bichromate de potassium* (Fig. 2 e). L'aspect est variable suivant le degré d'acidité, mais sous l'action de ce fixateur *une trame protoplasmique réticulaire* est toujours *grossièrement apparente*. A mesure que le pH augmente, la trame devient de plus en plus épaisse et irrégulière jusqu'à finalement être détruite.

*L'acide chromique* (Fig. 5) a une action comparable.

*L'acide picrique* seul, ne nous a pas donné de bons résultats mais il conserve souvent un réticulum hyaloplasmique bien ordonné, qui peut être même plus fin que celui de l'acide chromique.

*L'alcool* met en évidence une trame lâche très ajourée, à mailles fines, véritable dentelle, dans le hyaloplasme des polynucléaires et des monocytes (Fig. 3 c), mais une structure grossièrement fibrillaire dans les plaquettes (Fig. 6).

c. Les effets opposés, fins et épais, de ces deux grands groupes de fixateurs représentent des *extrêmes*, alors que les *mélanges fixateurs* réalisent des *aspects intermédiaires* où une trame cytoplasmique est toujours nettement visible, plus ou moins fine, suivant le pouvoir d'action de l'un ou de l'autre de ses constituants.

Ainsi, les mélanges à *base d'acide osmique* subissent l'influence «affinante» de celui-ci (Flemming, Benda, Meves, Champy). Le liquide de Champy en particulier (Fig. 2 b) réalise des aspects fins où se fait sentir nettement l'influence prédominante de l'acide osmique sur les autres constituants, tandis que ceux-ci sont sans doute responsables de l'aspect réticulé qui est partout retrouvé. Les images sont particulièrement satisfaisantes à cause de la netteté du réseau dont les éléments orientés avec évidence permettent facilement une étude simultanée de la trame elle-même et des éléments lipidiques: gouttelettes de graisse, mitochondries, appareil de Golgi.

Cette même action «affinante» peut être retrouvée dans les mélanges *à base de formol* (Orth, Müller-Formol, Regaud, Bouin): Le liquide d'Orth illustre particulièrement cette notion de l'influence prépondérante d'un liquide (formol) dans un mélange; le réseau très grossier de Müller seul (Fig. 2 f, 3 d) est transformé en un réseau fin. De même le Regaud sans doute sous l'influence du formol donne aussi des images fines et satisfaisantes. Et, si *l'acide picrique* seul abîme souvent les cellules, additionné au formol dans le *liquide de Bouin*, les résultats sont bien plus constants.

Dans les mélanges à *base de sublimé*, l'addition d'alcool, comme dans le *Liquide de Schaudinn*, ne semble pas avoir d'influence nette; les images sont semblables à celles du sublimé seul; de même l'acide acétique dans le *Mélange de Lang* a une influence faible; ce sont des images de sublimé moins fines et plus grossières, avec un réseau plus apparent.

Les aspects sont intermédiaires, généralement homogènes avec un réticulum bien visible pour les *Mélanges* de Heidenhain, Zenker, Helly (Fig. 7 a) et Maximow (Fig. 2 c).

Parmi les fixateurs à base de *bichromate de K*, le liquide de Tellyesnicsky nous a donné des résultats irréguliers; le hyaloplasme peut présenter des aspects alvéolaires et grossièrement spongieux comme si le bichromate était employé seul.

Le liquide de Müller a sur le hyaloplasme le même effet que le bichromate; la trame est plus épaisse encore et le relief toujours aussi marqué (Fig. 2 f et 3 d).

Notons encore l'action d'un groupe de substances rarement employées comme fixateurs jusqu'ici:

*L'acide phosphotungstique* et *phosphomolybdique*, *l'acétate d'uranium* et *le chlorure de platine* ont été utilisés dans l'espoir d'obtenir une imprégnation élective aux métaux lourds des fibrilles protoplasmiques, procédé déjà employé par Bessis et Bricka. Les résultats sont, dans l'ensemble, peu satisfaisants. Employés en solution à 1 %, ils ont une action nettement homogénéisante comme l'osmium. Le cytoplasme est entièrement imprégné par la substance et absorbe au maximum la lumière électronique.

Ainsi, tandis que l'on peut presque toujours suivre une trame de base dans la structure cytoplasmique, on voit que chaque fixateur est capable de la modifier dans son aspect d'une façon qui *est particulière à chacun d'eux. Le fixateur «crée» une image. Il est responsable en partie de cette image et de la structure ainsi révélée.* De ceci, nous pouvons tirer une conclusion d'ordre pratique intéressant le choix d'un fixateur pour un travail donné.

En effet, il ne s'agit pas toujours seulement d'obtenir une *bonne conservation*

de l'ensemble d'une cellule; celle-ci nous est assurée par certains des fixateurs classiques (acide osmique, formol) et, à ce point de vue, nous n'avons rien à ajouter mais on peut désirer *l'image la plus propice* à l'étude que l'on se propose. Ainsi, le fixateur employé avec prudence peut remplacer dans une certaine mesure le colorant qui fait défaut en microscopie électronique.

Une trame plate, effacée, telle que la réalise l'acide osmique, ne sera pas toujours préférable à une trame réticulée fine qui joue le rôle de «toile de fond» indiquant l'orientation de l'ultrastructure et sur laquelle se détacheront avec relief des éléments divers. Il n'est pas exclu que des fixateurs considérés comme «mauvais» puissent rendre service dans des cas précis (Fig. 5).

Donc d'une part certaines structures labiles dépendent presque entièrement de l'action du fixateur utilisé (*taille* des *grains* et des *fibrilles* mis en évidence), d'autre part une *orientation générale* de cette ultrastructure persiste dans la plupart des cas, et elle est *diverse suivant l'espèce cellulaire examinée.*

Sous l'influence d'un même fixateur, un monocyte, un polynucléaire, une plaquette, éléments de souches différentes, paraissent avoir un cytoplasme dissemblable, tant par la qualité des unités élémentaires qui le constituent que par la disposition d'ensemble de celles-ci.

En effet, le réticulum des *monocytes* et des *polynucléaires* est toujours plus fin que celui des *plaquettes.* Les aspects fibrillaires y sont moins grossiers. Dans le *monocyte*, l'orientation des éléments de la trame manque complètement ou est souvent circulaire, centrée par le noyau.

*Les polynucléaires* (Fig. 1 a, b, 7 a, b, 8 b) peuvent présenter une «polarité» nette, de part et d'autre du noyau. Il se forme d'un côté de fins pseudopodes de rétraction qui paraissent rayonner à partir d'un centre situé à la périphérie de la cellule (Fig. 1 a). Le hyaloplasme de cette région est lui aussi très nettement orienté: ses fibrilles ou bien irradient à partir de ce centre (Fig.

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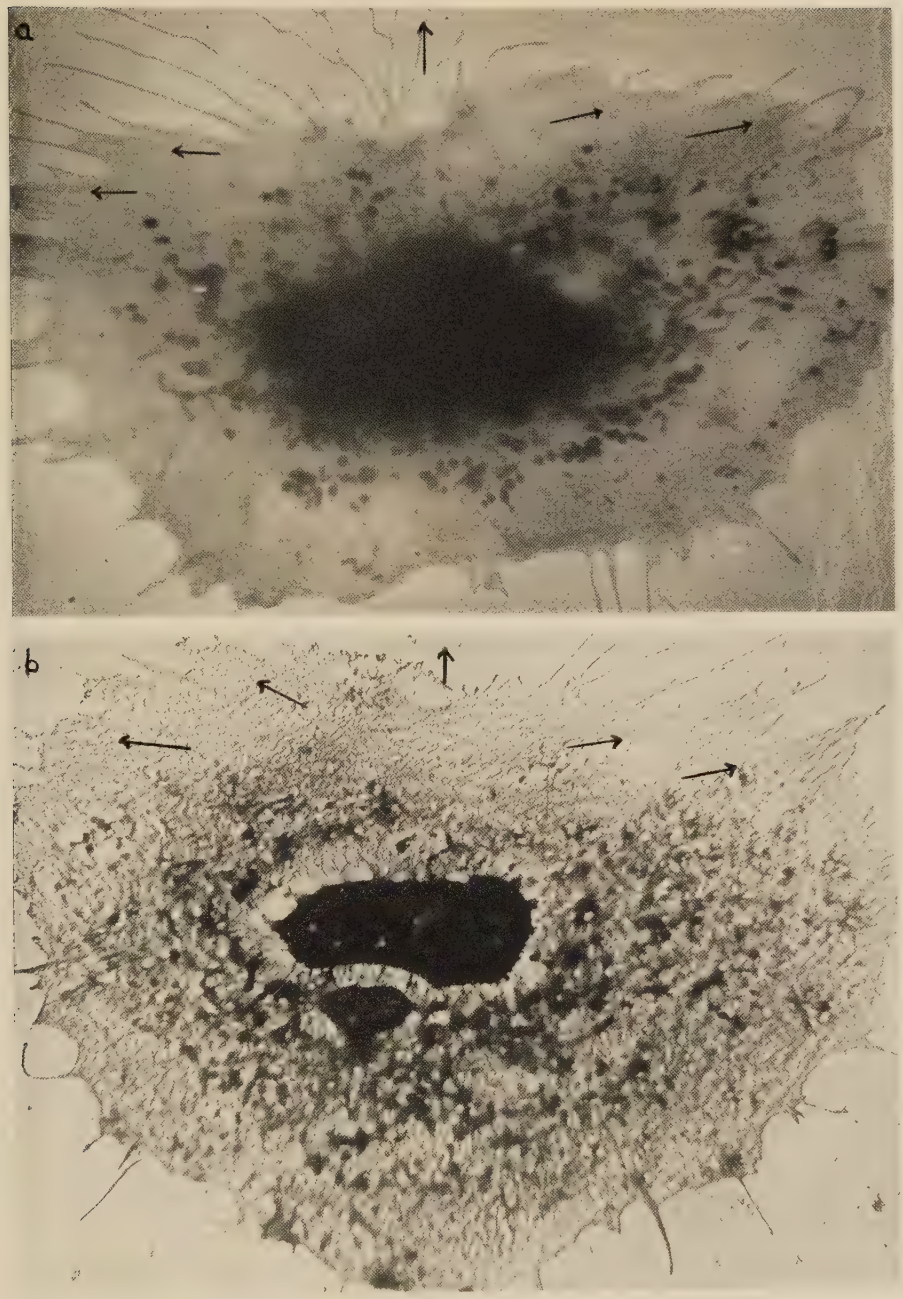
*Fig. 1.* — a) Polynucléaire neutrophile typique fixé à l'acide osmique 2%. — Gross. 3 800 ×.
Le cytoplasme est fin et homogène; la «polarité» de la cellule est nette avec la disposition particulière de pseudopodes et de fines fibrilles protoplasmiques à peine visibles mais indiquées par les flèches. La masse sombre centrale correspond au noyau. Tout autour sont disséminés les granulations spécifiques et le chondriome. En général, ces granulations sont beaucoup plus importantes dans la zone qui n'est pas «orientée».

b) Polynucléaire fixé au Zenker, puis digéré à la désoxyribonucléase pendant 4 h. — Gross. 3 800 ×.
Trame »macérée» grossièrement fibrillaire et réticulée du cytoplasme, mais montrant mieux que l'élément ci-dessus 2 zones différentes: l'une, en bas, sans orientation, où sont rassemblées les granulations; l'autre, en haut, où les fibrilles protoplasmiques sont orientées suivant le sens des flèches.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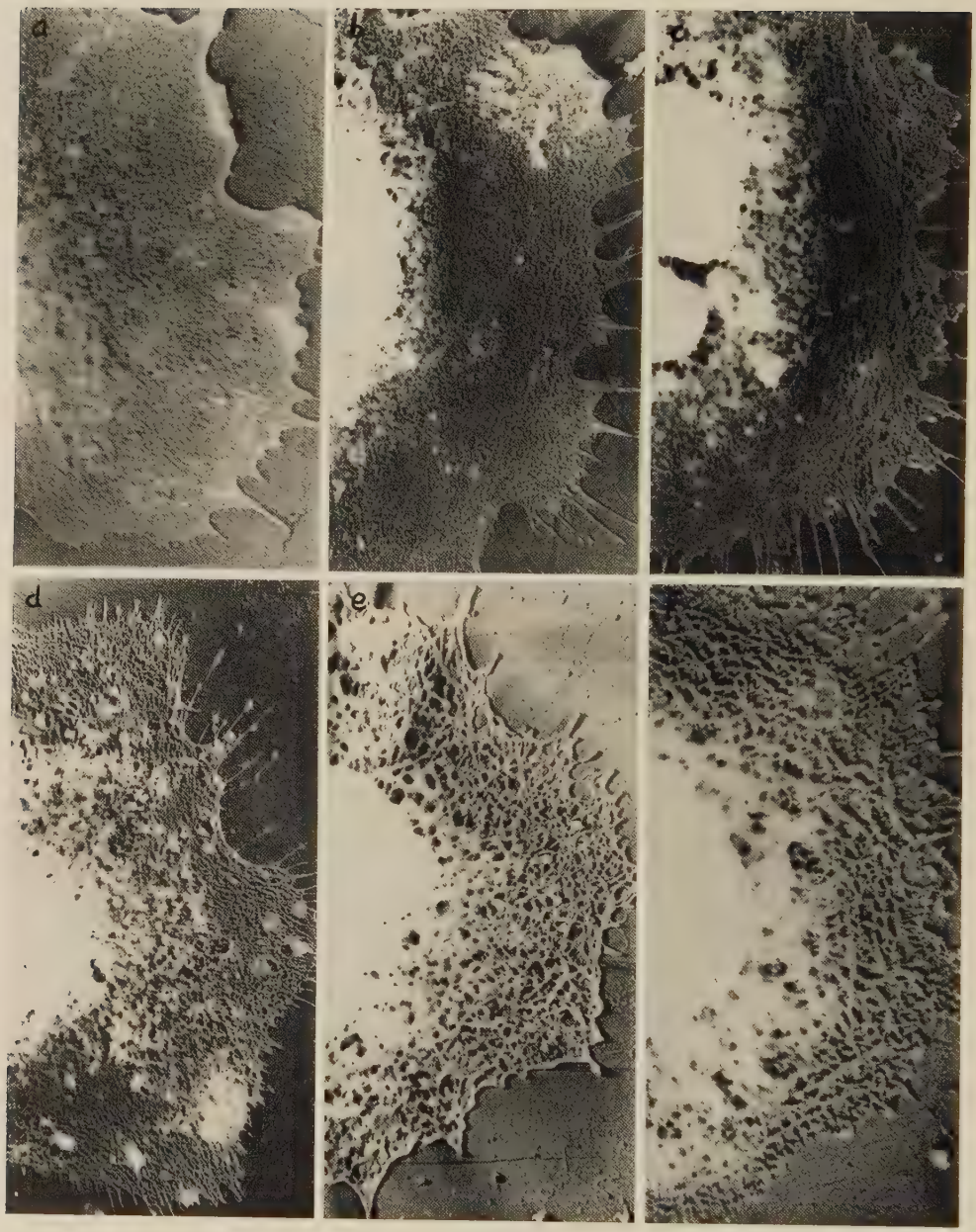



*Fig. 2.* — Portions cytoplasmiques de *polynucléaires* classées de manière à montrer l'influence «homogénéisante» des fixateurs du premier groupe (a, sublimé — b, Champy — c, Maximow) par contraste avec celle «grossissante» des fixateurs du deuxième groupe (d, Bouin — e, bichromate — f, Müller). On peut voir nettement la trame cytoplasmique et son épaississement progressif. Gross. entre 3 700 × et 4 500 ×.

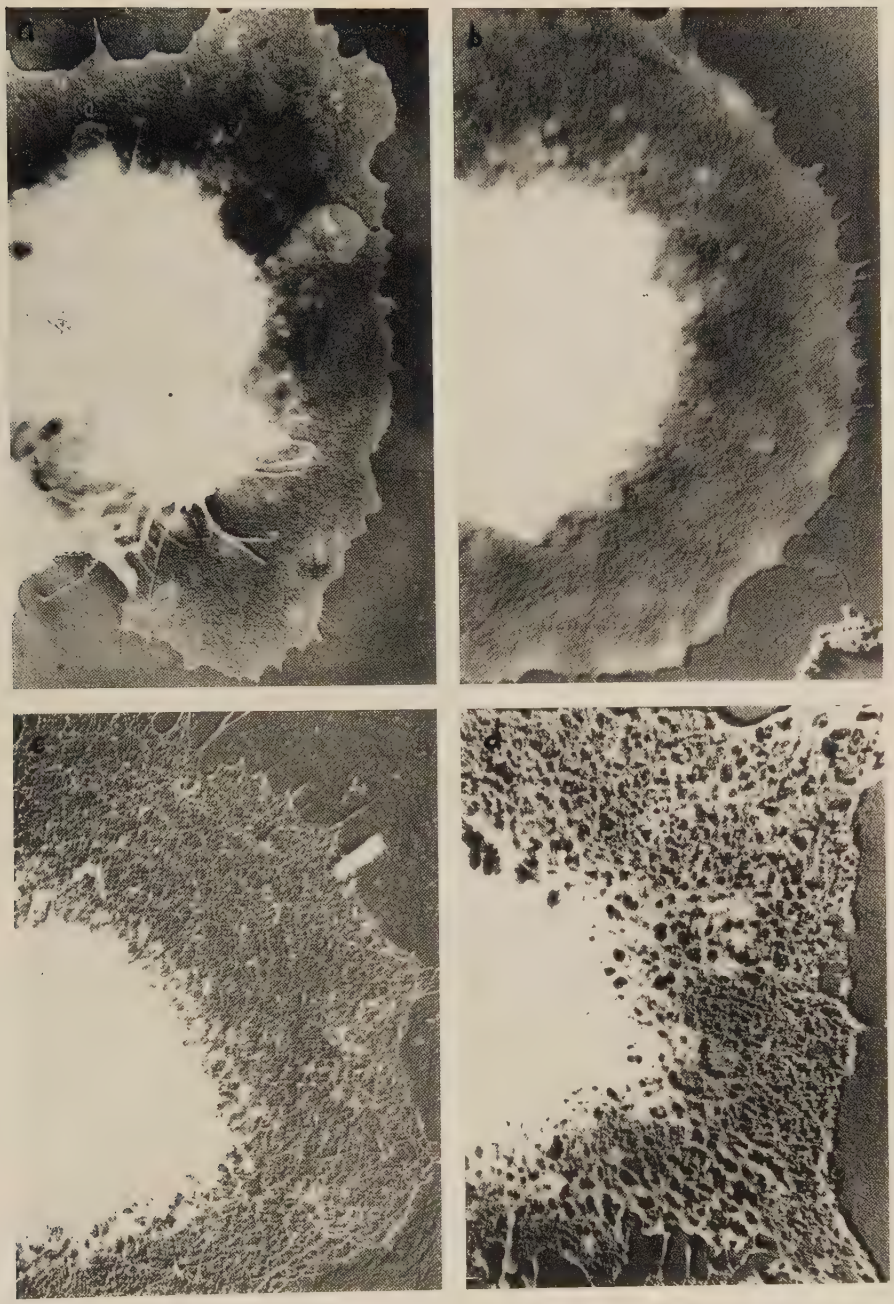



*Fig. 3.* — Cytoplasmes de *monocytes*.

En haut, cytoplasme fin et homogène des fixateurs du premier groupe — a, acide osmique — b, formol.

En bas, cytoplasme «réticulé» des fixateurs du deuxième groupe — c, alcool — d, Müller.

L'orientation circulaire de la trame ultrastructurale par rapport au noyau ne peut être observée sur ces éléments. Gross. entre 4 500 × et 5 200 ×.

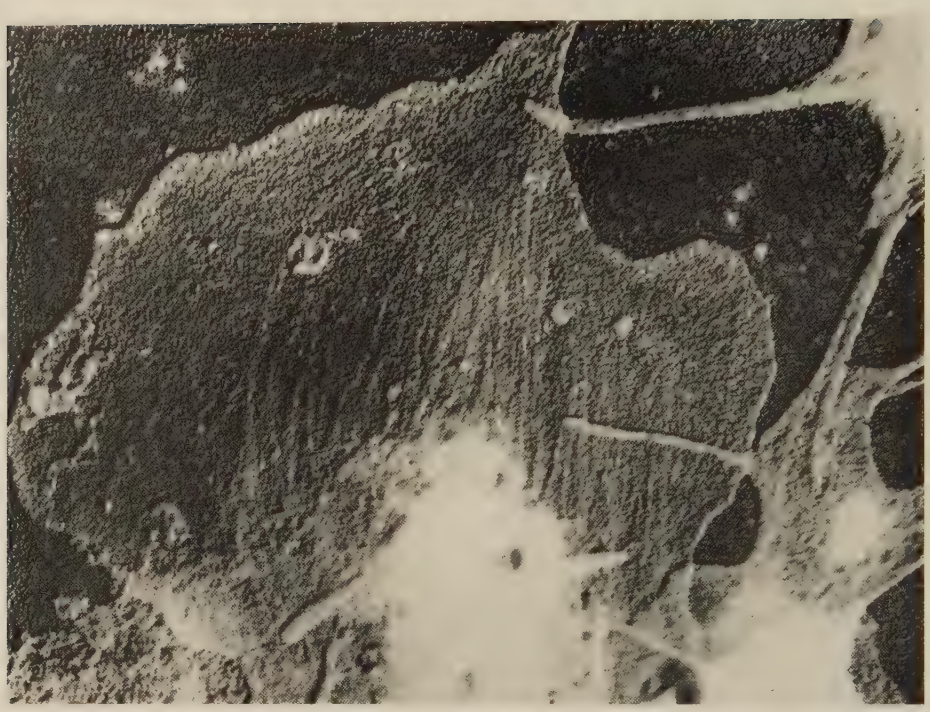


*Fig. 4.* — Portion d'une *plaquette* fixée à l'acide osmique 2 %. Gross. 12000 ×. Autre exemple de hyalomère «homogène». Mais déjà apparaît presque effacée une striation perpendiculaire par rapport au granulomère et typique pour un grand nombre de plaquettes.

7 a et b) ou bien sont parallèles au grand axe de la cellule (Fig. 1 b). Cette zone est tout à fait libre d'inclusions, qui sont groupées au contraire dans la région opposée, réticulaire et non ordonnée.

Les *plaquettes* enfin (Fig. 4, 5, 6, 8 c) ont un hyaloplasme beaucoup plus «résistant» que les autres cellules. Leur structure apparaît plus complexe; il existe parfois plusieurs plans, chacun formé de fibrilles assez épaisses qui s'entrecroisent sous des angles divers (Fig. 5), en rayonnant à partir du granulomère, ou en décrivant des courbes à convexité périphérique (Fig. 5). Nos images de plaquettes sont tout à fait semblables à celles obtenues par Bessis et Bricka (5, 6).

En conclusion, la différence ultrastructurale entre les éléments étudiés provenant de trois souches cellulaires distinctes, nous semble être frappante. Ces faits ont pu être mis en évidence plus facilement par l'utilisation de fixateurs très divers.

*Fig. 5.* — Plaquette fixée à l'acide chromique 1 %. Gross. 7500 ×. Image type de l'action d'un fixateur du deuxième groupe; la trame épaissie «révèle» et permet de bien voir les fibres radiaires et arciformes s'intriquant sur plusieurs plans. Exemple de la complexité de l'ultrastructure des thrombocytes.

## DISCUSSION

*Le Problème des Fixateurs.* Lorsque nous avons classé les fixateurs en deux grands groupes; ceux qui homogénéisent l'ultrastructure du hyaloplasme, ceux qui grossissent cette ultrastructure, nous avons adopté une classification purement morphologique qui nous semblait répondre à un

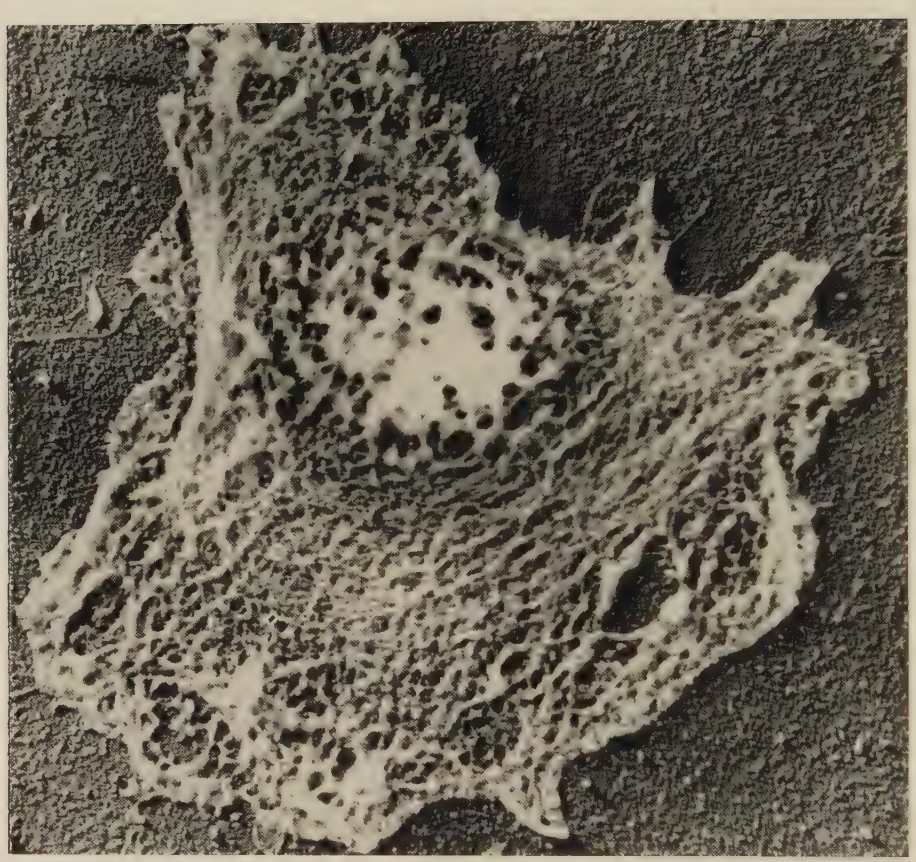


*Fig. 6.* — Plaquette fixée à l'alcool éthylique 100 %. Gross. env. 12000 ×. Trame épaisse, mais l'arrangement circulaire d'un faisceau fibrillaire reste encore bien visible. Pourtant, l'idée qu'on se trouve déjà en présence de gros artéfacts vient facilement à l'esprit.

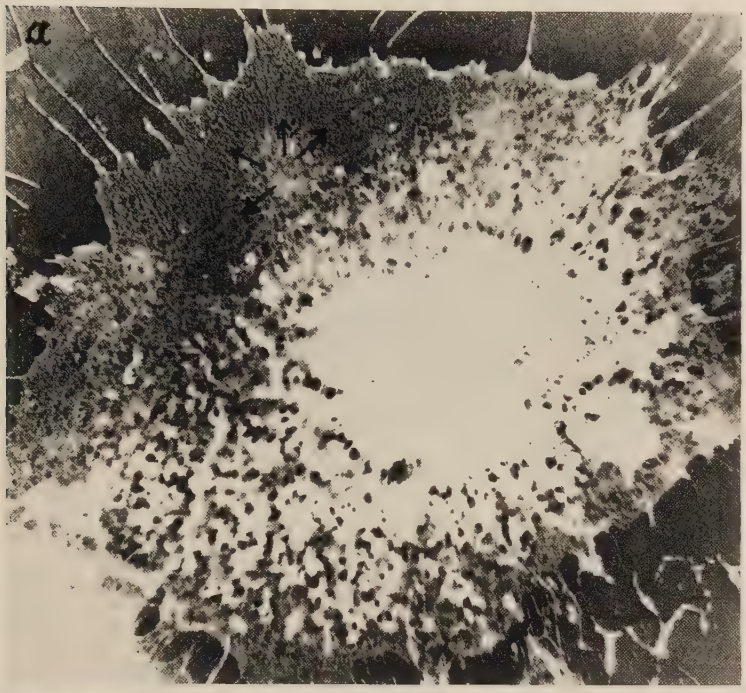
*besoin pratique* en microscopie électronique, où il est plus important de connaître les «qualités morphogènes» identiques ou voisines de divers fixateurs, que leurs propriétés chimiques.

Si l'on compare cette classification à celles plus courantes basées sur les propriétés chimiques des fixateurs, on ne peut expliquer que des sub-

*Fig. 7.* — a, Leucocyte fixé au Helly. — Gross. 5700 ×.

La «polarité» de la cellule est nettement visible, en bas la zone des granulations, en haut la zone hyaloplasmique libre d'inclusions où les flèches indiquent un «centre d'orientation» des fibrilles cytoplasmiques. Début de formation de pseudopodes.

b) Vu à un fort Gross (11300 ×). 2 «centres d'orientation» à partir desquels rayonnent les fibrilles hyaloplasmiques. Le cytoplasme «macéré» a été fixé au Zenker puis soumis à l'action de la désoxyribonucléase (ces cellules étaient destinées à une étude des noyaux).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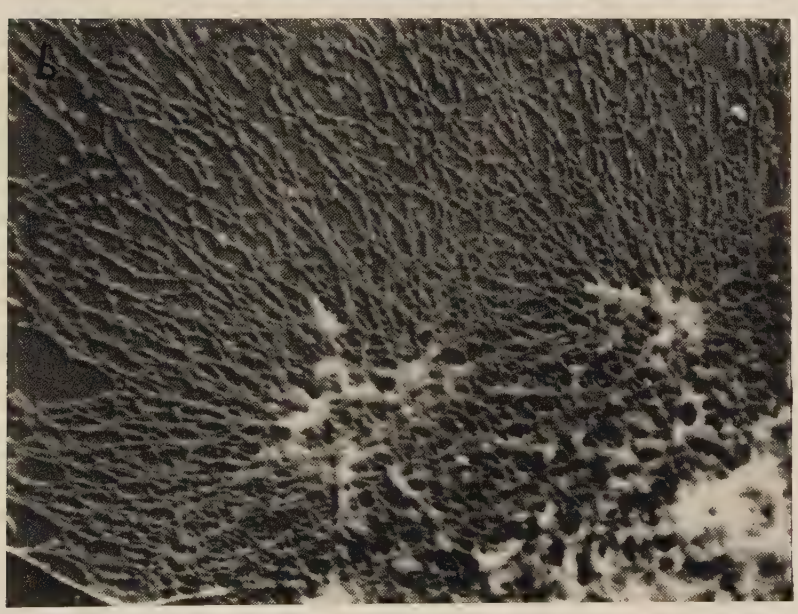
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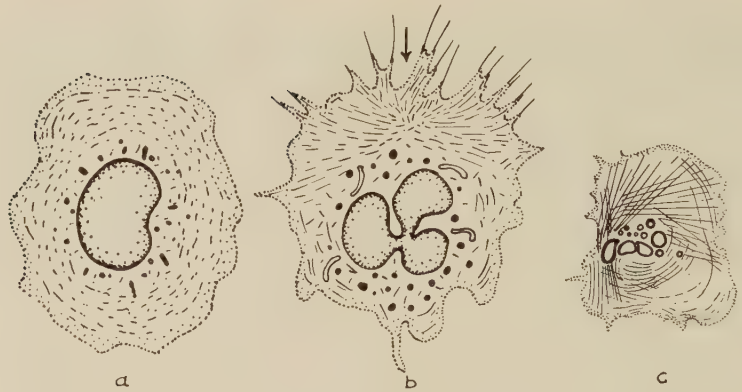


*Fig. 8.* — Dessin schématique montrant les principales dispositions que prennent les fibrilles hyaloplasmiques dans les monocytes (a) les polynucléaires (b) et les plaquettes (c).

stances à action chimique totalement différente comme l'acide osmique et le formol par exemple, aient une action très semblable et puissent se ranger dans une même catégorie.

L'explication de Zeiger (21–23) par contre, qui se base sur les théories de Frey-Wyssling (10) permet d'interpréter certains faits que nous avons constatés.

Cet auteur fait jouer un rôle prépondérant aux phosphatides dans le maintien de l'architecture submicroscopique de la cellule. Il les décrit comme formant une «façade lipoïdique» et suivant que cette façade est consolidée ou détruite, la charpente protéinique est ou n'est plus protégée et l'ultrastructure est ou n'est plus conservée. Il est ainsi amené à diviser les fixateurs en *Coagulants des protéines* — (alcool, acétone, acide acétique, acide picrique et sublimé) et en *Stabilisants des lipides* — (acide osmique, formol, bichromate de potassium).

Plus une substance stabilise les lipides, meilleure est son action fixatrice. L'aide osmique et le formol sont pour cette raison de bons fixateurs.

D'autres actions entrent également en jeu pour ces deux fixateurs (formation de petites particules de divers hydrates d'oxyde d'osmium comblant les mailles du réseau hyaloplasmique, réaction des groupements $NH_2$ des acides aminés avec le formol et création de ponts methyléniques («Vernetzung»).

De même les images de cytoplasmes fixés à l'acide acétique, à l'alcool, sont témoins de l'action destructrice de ces fixateurs vis à vis des lipides et des protéines.

Mais comment expliquer l'aspect du hyaloplasme fixé au bichromate de potassium qui appartient au groupe des «stabilisants des lipides» et dont l'image est une des moins homogènes et celui réalisé par le sublimé, qui appartient au groupe des «coagulants des protéines» qui nous apparaît au contraire, comme relativement homogène?

Peut-être, l'interprétation chimique de certaines images manquera-t-elle toujours, la réaction du hyaloplasme restant «globale»: grossissement de la trame ou rupture, quelle que puisse être l'action chimique et la déformation initiale, celle-ci demeurant au-dessous du pouvoir séparateur.

Néanmoins, la voie indiquée par Zeiger (21–23) mérite d'être suivie: c'est celle de *l'expérimentation exacte avec les fixateurs.* Ne nous fions plus à un seul fixateur mais d'après les images variées et *toujours plus ou moins artificielles* de fixateurs divers, déduisons l'architecture réelle d'une ultrastructure. Les artéfacts sont donc volontairement utilisés au service de la reconstitution morphologique.

Mais qu'entendons-nous par «artéfacts»?

Il ne s'agit bien entendu pas seulement de ces formations d'origine exogène dues le plus souvent à des précipitations surajoutées qui, comme en microscopie optique, sont faciles à bien connaître, *mais de toute modification structurale changeant la taille ou la forme d'une structure vitale.*

Ainsi, nous sommes amenés à nous demander s'il est vrai que l'image créée par un fixateur est d'autant plus proche de la réalité qu'elle est plus fine, comme on a l'habitude de le faire actuellement. N'existerait-il pas des artéfacts «d'effacement», «d'affinement», aussi bien que des artéfacts «d'épaississement» de la structure?

Une interprétation plus nuancée de ce mot, souvent lancé comme un verdict, s'impose. Les structures peuvent rester inchangées (*identité*) ou s'épaissir (*agrégation*), se déformer (*analogie*), s'amincir (*désagrégation*) ou totalement disparaître. D'autre part, une structure peut être déjà présente *in vivo*, mais à cause du degré d'hydratation rester invisible. Le fixateur en déshydratant joue ainsi le rôle de *révélateur* (*structures démasquées*, Zeiger). Ou bien, les structures n'existent que *virtuellement in vivo* (ex.: molécules libres rangées le long d'une ligne de force de tension superficielle). En les coagulant sur place, le fixateur peut créer une structure cohérente. Enfin, des images de *corps surajoutés* représenteraient les artéfacts au sens strict du mot.

*Le Problème de l'Ultrastructure.* Cette étude, enfin, nous amène à discuter la *structure réelle*, l'élément stable du cytoplasme qui résiste le plus à la déformation des fixateurs les plus divers.

Est-ce que la mise en évidence d'un réticulum hyaloplasmique nous autorise d'avance à conclure que le cytoplasme vivant est fibrillaire et non pas granulaire? Comme Bretschneider (7, 8) l'a montré pour la lymphe, et comme nous l'avons constaté pour le sérum sanguin, la *coagulation de protéines,* suspendues dans un liquide peut *créer artificiellement un réseau.* Mais *celui-ci n'est pas ordonné.* Notre hyaloplasme a souvent une forme typique (pseudopodes leucocytaires, plaquettes) que l'on ne peut s'expliquer qu'en admettant la préexistance de fibrilles ce qui est en accord avec d'autres observations (biréfringeance, micrurgie). Mais on ne peut certes pas affirmer que des formes granulaires ne coexistent pas.

Quels sont ces facteurs qui déterminent l'architecture ultrastructurale?

*Le noyau,* d'une part, la *forme cellulaire* d'autre part, *semblent déterminer cette disposition circulaire des fibrilles cytoplasmiques* dans le *monocyte* et dans le *polynucléaire* arrondi. Si les cellules s'allongent, les fibres intra-hyaloplasmiques prennent immédiatement une disposition parallèle à l'axe de la cellule. Ce fait a déjà été constaté par Pollister (15), qui croit que l'orientation des mitochondries est un indice de l'orientation hyaloplasmique. D'autre part, Monné (12–14) conclut d'après des observations faites au microscope polarisant que les chaînes de la trame moléculaire prennent une disposition tangentielle par rapport au noyau. Enfin, Schmitt (18) observe une biréfringence de forme temporaire de la partie du cytoplasme qui s'étale. Nos micrographies électroniques confirment donc de façon très nette les observations de ces auteurs.

On peut d'ailleurs facilement réaliser une disposition structurale analogue en laissant s'écouler une masse visqueuse (celloïdine à 10 %) contenant des particules légères et allongées (cheveux coupés à 2–5 mm) sur un «noyau» artificiel en paraffine reposant sur une plaque de verre. En laissant s'étaler ce mélange autour du noyau, on observe une disposition circulaire des poils. En inclinant la vitre d'un côté, on obtient un large «pseudopode» où les particules s'orientent parallèlement à son axe. L'orientation de la «structure» de ce modèle artificiel présente des analogies indéniables avec l'orientation de la trame ultramicroscopique d'une vraie cellule.

Mais d'autres polynucléaires, nous l'avons vu, sont: «polarisés». Ils émettent des pseudopodes filiformes à la base desquels on peut trouver un ou même plusieurs *centres d'orientation* (Fig. 7 a, b), comme Monné (12–14) les a déjà observés dans des cellules à cytoplasme large bien étalé. Le centrosome fonctionnerait ainsi comme «sekundäres Schichtungszentrum». Mais de tels centres pourraient se former ailleurs.

Ces «dispositifs» ne sauraient s'expliquer de façon aussi simple que les précédents et encore moins ceux des *plaquettes.* On ne peut guère concevoir

un modèle mécanique qui permettrait d'imiter cette architecture par le simple écoulement d'un liquide visqueux.

Quelle que soit la diversité d'interprétation des ces faits, la complexité structurale des différentes cellules pose des problèmes fondamentaux:

— Celui du *rapport entre ultrastructure et fonction cellulaire* d'une part.

— Celui d'une *spécificité structurale,* d'autre part.

Et, à ce dernier point de vue, il reste ce fait: le *hyaloplasme optiquement vide et homogène peut, après fixation, avoir une architecture bien définie et spécifique dans certaines souches cellulaires.* Il ne nous semble pas exclu qu'un grand nombre d'autres cellules aient leur propre ultrastructure protoplasmique. Il existe des dispositifs «simples» ou «compliqués», mais le matériel de base reste le même: fibrilles fines à aspect parfois granulaire, de tailles diverses ordonnées et parallèles ou désordonnées en réseau. Ainsi, Bretschneider (7, 8) décrit, dans le méristème *d'Allium,* un exemple de cytoplasme, «simple» à réseau si régulier qu'il peut même parler d'un système presque hexagonal de «point de jonction» avec une périodicité déterminée et, pour lui, la microscopie électronique confirme ainsi la théorie de Frey-Wyssling (10).

En ce qui nous concerne, nous ne saurions être aussi affirmatifs. Si la théorie des «points de jonction» appliquée à l'étude de l'action des fixateurs (21–23) nous a rendu de grands services, nous ne croyons pas que l'on puisse l'adopter sous une forme aussi schématique. L'exemple seul de ces trames complexes que nous venons de décrire à côté de ce réseau simple décrit par Bretschneider (7, 8) est, nous semble-t-il, démonstratif. On ne peut certes pas affirmer qu'une trame moléculaire à réseau régulier n'est pas le substratum *originel* de tous ces faisceaux fibrillaires qui s'entrecroisent et qui caractériseraient une cellule plus évoluée. Mais il n'en reste pas moins que le microscope électronique ne nous permet pas de voir une trame moléculaire de base, telle que la postule Frey-Wyssling (10). *Les fibrilles hyaloplasmiques observées restent toujours bien au-dessus du pouvoir séparateur du microscope électronique.*

La presque totalité des «unités» les plus fines, les «leptones» (7, 8), mesure encore 160 Å.

Fauré-Fremiet et Bessis, Bairati (1, 2, 9) et nous-mêmes, n'avons pu voir que des structures bien au-dessus de l'échelle moléculaire, nos éléments les plus fins dépassant au moins encore 20 à 30 fois l'épaisseur d'une chaîne polypeptidique simple.

Deux interprétations de ces faits sont possibles:

Ou bien l'ultrastructure hyaloplasmique est toujours d'ordre *supramolé-*

*culaire* et les unités de la trame comprennent toujours des *faisceaux* de chaînes polypeptidiques. Cette constatation viendrait à l'appui de l'opinion de Monné (14).

Ou bien toutes les observations faites au microscope électronique jusqu'ici, ne montrent que des *structures d'agrégation* d'un réseau vraiment moléculaire à son origine, mais «grossi», «condensé» par la technique de préparation.

Ce dernier point ne peut être entièrement résolu. Mais en tous cas, nous serions amenés à admettre, étant donné la grande *Variété* de diamètres des fibrilles protoplasmiques de certaines cellules, que des faisceaux plurimoléculaires coexistent très souvent *in vivo* avec des chaînes polypeptidiques simples.

Quant aux détails de forme et de mode de liaisons unissant les unités structurales qui opposent les théories, nous ne pouvons les résoudre. Par exemple, le schéma que propose Wrinch, de chaines flexibles composées de molécules protéiniques sphériques et rigides de 30 à 50 Å, peut donner les mêmes images que les faisceaux de chaînes polypeptidiques de Frey-Wyssling (10).

En conclusion, la microscopie électronique met en évidence sur des cellules fixées des structures cytoplasmiques semblables ou même identiques à celles postulées par les théories qui n'étaient basées que sur des résultats de recherches indirectes. Mais d'autres problèmes restent à préciser qui dépassent les moyens de cet instrument et de cette technique.

## RÉSUMÉ

Nous avons étudié au microscope électronique l'action des fixateurs sur l'ultrastructure du cytoplasme de leucocytes et de thrombocytes du sang humain normal.

1. La plupart des images obtenues sous l'influence des différents fixateurs sont comparables, quel que soit le mode d'action de ceux-ci. Elles mettent en évidence une trame hyaloplasmique réticulée, ou fibrillaire, plus rarement granulaire, de dimensions submicroscopiques mais supramoléculaires.

2. Les fixateurs peuvent être classés en *deux groupes:* ceux qui affinent la trame ou même l'effacent complètement, et ceux qui la grossissent à des degrés divers allant jusqu'à la rupture totale de l'architecture hyaloplasmique. Les mélanges fixateurs donnent des images intermédiaires suivant que le liquide prédominant appartient à l'un ou l'autre groupe.

3. La plupart des fixateurs «créent» ainsi une image qui leur est particulière,

mais ils respectent en général l'orientation des fibrilles ou du réseau qui est propre à la cellule.

4. Cette orientation peut être conditionnée par des forces purement mécaniques (tension de surface, courants cytoplasmiques lors de l'étalement de la cellule), mais d'autres facteurs semblent intervenir également.

5. La spécificité de l'ultrastructure hyaloplasmique des éléments sanguins ne fait pas de doute. On peut supposer qu'un grand nombre d'autres cellules ont, elles aussi, leur architecture propre.

6. Les travaux de microscopie électronique concernant l'ultramorphologie du cytoplasme ne nous paraissent pas confirmer d'une façon absolue la théorie des points de jonction de Frey-Wyssling, mais ne l'excluent nullement. Elle reste une bonne hypothèse de travail, si l'on se garde de l'appliquer trop schématiquement, car l'ultrastructure du hyaloplasme montre une très grande variété de dimensions et d'orientation, spécifique pour certaines souches cellulaires.

**SUMMARY**

The action of about 30 usual fixatives on the ultrastructure of the leucocytes and the thrombocytes of human blood was systematically studied.

It was found that in spite of their different mode of action the pictures were comparable showing mostly a fibrillary cytoplasmic network of submicroscopical dimensions.

The fixatives were divided into 2 groups according to the dimensions of this network: those which "reduce" or even completely suppress (e.g., osmium acid, formol), those which on the contrary exaggerate it more or less (e.g., chromic acid, picric acid, alcohol). Fixative mixtures show intermediate pictures.

A particular picture is thus "created" by the different fixatives but a general orientation of the fibrils or of the network of the cell is always respected.

This orientation can be conditioned by purely mechanical forces such as surface tension, intraprotoplasmic streams during the spreading of the cell but other factors play a role.

It appears certain that there is a definite specificity in the cytoplasmic ultrastructure of the different kinds of blood cells studied and one can imagine that many other kinds of cells have also their own architecture.

The electron microscopic studies do not appear to confirm in its totality Frey-Wyssling's "Haftpunkt-Theorie" but it remains a useful work-hypo-

thesis if not too schematically interpreted: the cytoplasmic ultrastructure shows a very great variety of dimensions and orientations specific for some cellular strains.

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# THE BASIS FOR THE SPECIFICITY OF METHYL GREEN STAINING

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In his paper, "The specificity of the methyl green-pyronin stain for nucleic acids", Taft (12) attacks the thesis that methyl green and pyronin specificity depend upon the state of polymerization of the desoxyribosenucleic acid (DNA). This thesis, originally presented by us at the Cold Spring Harbor Symposium in 1947 (2), permitted Pollister and Leuchtenberger (11), and Vendrely and Vendrely (14) to interpret cytological observations on heated and degenerating tissues. The detailed data on methyl green-pyronin staining of nucleic acids in test-tubes and in nuclei, in support of this theory, were presented in publications in 1949 and 1950 (3, 4, 7, 8, 9).

Taft states that it is unlikely that polymerization of DNA is a factor in methyl green staining, because he failed to detect any loss of methyl green affinity by chromatin after treatment of tissues at pH 3.0 and 11.7 at 1° C and 24° C for unstated periods of time. It is stated that such treatment of tissues may be expected to depolymerize the DNA in view of the observations of Gulland and Jordan (1) on the behavior of a purified sample of highly polymerized DNA. These authors found a prompt reduction in viscosity of a solution of DNA at pH below 5 and above 11. Gulland and Jordan's "depolymerization" is reversed (even after exposure to pH 12.5) by readjusting the pH to neutral (resembling, in this respect, the reversible effect of electrolytes on the viscosity of DNA solutions), and is interpreted by them as due to the breaking of hydrogen bonds between adjacent bases, and not to the rupture of internucleotide linkages. It is thus different from the *irreversible* degradation produced by heat, desoxyribonuclease, or prolonged treatment with strong alkalies, which cause rupture of inter-nucleotide linkages. In fact, we have found DNA to remain highly polymerized even after the application of 0.1 HCl to nucleohistone (for the removal of histone) as determined by the fibrous nature of the pricipitated DNA, the high viscosity on redissolution,

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and the undiminished affinity for methyl green (4, 9). For these reasons, it is doubtful that Taft's procedure did indeed depolymerize DNA, and no data are presented to demonstrate depolymerization. The fact that this treatment of tissues produced some changes (considerable loss of pyroninophilia) is not surprising, particularly in alkaline solution, which is known to remove ribosenucleic acid (RNA) from tissues (10) and provides no evidence as to the effect on DNA. Prolonged soaking in alkaline solution might also be expected to remove some DNA, although 70 per cent alcohol is hardly a good solvent for either nucleic acid.

Several other observations made by Taft call for some comment. The statement is made that treatment of tissues at pH 3 and pH 11.7 markedly reduced the intensity of the Feulgen stain in the absence of effect on methyl green staining. This is rather surprising, since, in the course of the Feulgen reaction treatment with strong acid (1 N HCl) at 60° C is applied for 10 to 20 minutes (depending upon the method of fixation) without loss of stainability. Since Taft's observations were only qualitative (visual), comparison of the intensity of methyl green and Feulgen staining could hardly be precise. Furthermore, methyl green was not applied by Taft in a manner which has been demonstrated to result in stoichiometric staining (3, 5, 7). This may also account for the slight staining of non-nuclear material observed by Taft in some tissues. The latter phenomenon may also be due to incomplete purification of the methyl green. We have never observed polychromatic staining (blue, blue green, green, *lavender*) reported by Taft when applying methyl green alone. It is our own practice to extract *solutions* of methyl green in acetate buffer (pH 4.0–4.2) with chloroform until the chloroform extract becomes completely colorless. Removal of the contaminating dyes (principally crystal violet) is likely to be more complete from a solution than from the dry stain mixture, in which occlusion may occur.

A number of Taft's observations may be interpreted in support of our theory. He noted, in confirmation of Unna (13) and Pollister and Leuchtenberger (11), that subjecting tissues to boiling water destroyed affinity for DNA without influencing the Feulgen stain. It should be remarked that the same effect has been observed on subjecting dry paraffin tissue sections to 60–65° C (5), and that such temperatures depolymerize DNA (4, 6, 8, 9). His failure to note differential staining by methyl green-pyronin of solutions of commercial RNA and DNA is supportive evidence, since with the exception of Worthington Laboratory's DNA, the currently available commercial nucleic acids are all very low molecular weight mixtures. The loss of stainability of isolated nuclei with methyl green after embedding in gelatin, referred to by Taft, may be a function of the temperature of embedding used, which is not stated.

We have found the affinity of DNA for methyl green to be so precise a

function of the state of polymerization of the former as to have succeeded in developing a quantitative method for the determination of desoxyribonuclease activity based upon this relationship (6).

Tests of the theory that polymerization determines DNA affinity for methyl green should be based upon the effect of *demonstrated* depolymerization of the DNA or its affinity for methyl green rather than upon surmised depolymerization.

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# THE METHYL GREEN-PYRONIN METHOD

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The differential staining of nuclei, nucleoli and cytoplasm by an aqueous mixture of methyl green and pyronin at a suitable pH is well known. Brachet (1) stained material, both treated and untreated with ribonuclease, with the mixture to demonstrate the localisation of ribosenucleic acid. Recently Kurnick (3) showed that when a mixture of methyl green and pyronin is used to stain polymerised desoxyribosenucleic acid, the methyl green stains selectively while, if used to stain depolymerised desoxyribosenucleic acid or ribosenucleic acid, it is the pyronin which stains selectively. It was believed that when the mixture was used to stain cells a similar selective staining occurred, the methyl green selecting the desoxyribosenucleic acid (DNA) and the pyronin the ribosenucleic acid (RNA). From this Kurnick developed a method for the quantitative staining of desoxyribosenucleic acid in cells by methyl green (4). Kurnick and Mirsky (5) showed this method to be based on a stoichiometric reaction between the dye and the polymerised desoxyribosenucleic acid. It seemed from this work that it should be possible to demonstrate polymerised desoxyribosenucleic acid by staining the cell with methyl green, and other basophilic materials by counterstaining with pyronin, since the action of the mixture was not competitive.

In the present study, roots of the broad bean *Vicia faba* were fixed for 24 hours in absolute alcohol. They were embedded in paraffin sectioned longitudinally at 7$\mu$, the paraffin was removed with xylol, and the sections were taken down through the alcohols to water. The methyl green stain was 0.25 per cent 'Revector' methyl green, N2, batch No. 02540, in 0.2 M acetate buffer, pH 4.2, purified by extracting several times with chloroform. The absorption curve of the purified dye agreed with that figured by Kurnick (3). The pyronin stain used in these studies was a 0.1 per cent solution of pyronin (Gurr, No. 447) in 0.2 M acetate buffer, pH 4.2.

In the standard methyl green-pyronin method as described e.g. by Darlington and La Cour (2), sections are stained with a mixture of methyl green and pyronin for about 30 minutes. Root sections were therefore stained for 30 minutes with the purified solution of methyl green, rinsed in distilled water,

and immersed in the pyronin solution for 30 minutes. Some sections were rinsed briefly in distilled water and examined; others were made permanent by blotting dry, rinsing in absolute alcohol, clearing in xylol and mounting in Depex. No trace of the original methyl green staining remained after this "counterstaining" with pyronin. To find whether the pyronin merely overlaid and masked the methyl green staining, sections were stained for 30 minutes with methyl green, rinsed briefly, counterstained for 30 minutes in pyronin, and subsequently immersed in 0.2 M acetate buffer, pH 4.2. After a short time all colour was removed from the tissue.

Similar sections were stained with methyl green according to Kurnick's schedule: after treatment with 0.01 N hydrochloric acid for 5 minutes they were washed in 0.2 M acetate buffer for 10 minutes after which they were stained in a refrigerator overnight in the purified methyl green solution. They were then washed in 0.05 M acetate buffer, pH 4.2, for 10 minutes and transferred to fresh 0.05 M buffer solution for another 10 minutes to remove unreacted dye. The intensity of the staining was apparently not decreased even after 2–3 days in buffer solution. The buffer solution left only the nuclei stained intensely, their colour being blue. The slides were placed in a 0.1 per cent solution in 0.2 M acetate buffer, pH 4.2, of pyronin (Gurr, No. 447). After 2 minutes in stain the cells were reddish, and at the end of 4 minutes very little trace of the previous methyl green staining was visible. With 15 minutes staining the nuclei became deep red which strongly suggested that the methyl green had been displaced by the pyronin. When the slides were immersed in fresh 0.05 M buffer solution for 3 hours, however, the pyronin was completely washed out of the sections but the blue colouration of the nuclei reappeared as it was before the cells were counterstained with pyronin.

Preparations were then made in which the sections were stained with methyl green by Kurnick's schedule and counterstained for 30 minutes with a mixture of equal parts of the methyl green and the pyronin solutions. The nuclei were stained blue, nucleoli and cytoplasm red; the results compared very favourably with those obtained by more standard procedures, such as that given by Darlington and La Cour (2).

These experiments were repeated on newt tissue cultures grown from newt lung. The cultures were fixed in Ringer-formol for 20 minutes, stained with methyl green and subsequently counterstained either with pyronin, followed by destaining in buffer solution, or with the methyl green-pyronin mixture. Results exactly comparable with those described for the bean root sections were obtained, except that the nuclei were green instead of blue. Other

cultures were fixed in alcohol and stained with methyl green as before. The nuclei again stained green instead of blue, showing that the colouration was not due to different fixatives. It is therefore possible that the difference in staining reaction between the animal and plant desoxyribosenucleic acid may be due to differences in the desoxyribosenucleic acids of the two tissues.

The fact that a differential colouration could not be produced by staining with methyl green for a short time and then with pyronin, at first led to the view that the stains had to compete in order to differentiate between DNA and RNA. This would have raised doubts about the selective staining claimed by Kurnick, but the fact that, after prolonged staining with methyl green, the pyronin used as a counterstain can be removed by buffer solution to disclose the unaffected methyl green staining of the nucleus has shown that the pyronin does not drive the methyl green out of its combination with DNA, but merely overlies and obscures it. The uptake of pyronin by the nucleus might suggest the presence of RNA on the resting chromosomes. This is made less likely, however, by the results obtained by counterstaining methyl green-stained nuclei with methyl green-pyronin. After the chromosomes had been stained with the methyl green so that the DNA had combined with this stain, we should expect that when the cells were counterstained with the mixture of methyl green and pyronin the remaining basophilic substance on the chromosomes, if it were RNA, would stain with the pyronin of the mixture, as do the nucleolus and cytoplasm, whereas in fact it takes up more methyl green in preference so that the chromosomes are stained a much darker blue than before.

It is, of course, possible that there is a red component, due to pyronin, in this deepening of the blue colouration of the nucleus, especially in the case of the newt tissue culture, but if so, it is negligible compared with the red colouration of the nucleus when counterstained with pyronin alone. This suggests that when the nuclei are counterstained with the mixture the pyronin is less successful in its competition with the methyl green for these other nuclear basophilic sites than it would be expected to be if competing for RNA only.

In conclusion it is clear that Kurnick's method binds methyl green to nuclei more strongly than does a short treatment. Thus it is possible that while the standard method, employing a short exposure of sections to a mixture of the dyes, may depend on competition between the two dyes, Kurnick's method may be selective for polymerised nucleic acid. As Taft (6) has pointed out, however, pyronin staining cannot be taken as specific for RNA.

It should be noted that in this paper reference to a particular nucleic acid is made for convenience only. It does not imply either that the nucleic acid is free, or that it is present as a nucleoprotein.

## SUMMARY

It has been shown that when methyl green is used to stain DNA, as by Kurnick's method, it binds firmly and cannot be washed off by immersion in a buffer solution. When tissue is stained with methyl green for a short time, however, either alone or mixed with pyronin, no strong binding is produced between the methyl green and the DNA. It is suggested that although Kurnick's method may be selective for DNA, the differential staining of cell structures produced by short treatment with methyl green-pyronin is due to competition.

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# THE EFFECT OF ULTRASONICS ON MITOSIS

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Since the pioneer work of Wood and Loomis (24), many authors have described the lethal effects of ultrasonic radiation applied through a water medium to unicellular and small multicellular organisms. These investigations are fully reviewed by Dognon and Biancani (7). As these authors conclude, the cellular disruption caused during ultrasonic treatment is due to the mechanical effect of cavitation in the aqueous medium surrounding the cells. There are, however, few examples of the biological effects of ultrasonics on material which survived treatment, although Dognon and Biancani (7) and Schmitt and Uhlemeyer (20) have shown that ultrasonics can cause the chloroplasts of certain water plants (viz. *Elodea* and *Spirogyra*) to be agitated violently within the living cells. In the author's experience these effects can occur at ultrasonic intensities insufficiently great for cavitation to be possible.

The present work is concerned with the effects of ultrasonics on the contents of the living cell nucleus with special reference to mitosis. In view of the remarkable chromosome aberrations which can be induced in living material by treatment with ionising radiations and certain chemical reagents, the cytological effects of any new kind of radiation must be regarded as unpredictable. Any breakage of chromosomes which could be achieved by mechanical means within the living cell nucleus would allow interesting comparisons to be made with similar breaks caused by other agents. The object of this work was to determine whether ultrasonics can produce cytogenetic effects.

Comparison of the results obtained by previous workers using ultrasonics in the biological field is made difficult by the fact that the physical conditions under which the experiments were performed are seldom precisely stated. In many cases the ultrasonic intensity applied to the specimen was not measured, and adequate conditions of temperature-control were seldom achieved. In cases where multicellular tissue was treated the thermal heating

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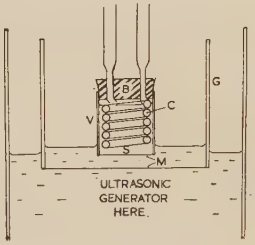
arising from the absorption of the ultrasonic energy in the tissue was often the dominant lethal factor (12).

In the present work all specimens were treated with uniform beams of ultrasonics of known intensity. The ultrasonic generator which was specially developed for the treatment of biological material, and the calorimetric method used to measure the ultrasonic intensity, are described by Selman and Wilkins (18). The arrangement of apparatus used when treating biological material is illustrated in Text-fig. 1. The specimen was placed at S in about 5 c.c. of the appropriate aqueous medium contained




Text-fig. 1. Diagram of ultrasonic treatment vessel.

in a glass tube V, 2 cm in diameter, with a cellophane window M forming the bottom. The tube fitted over a rubber bung B through which passed the cooling coil C, made in the form of a helix of thin closely-wound glass tubing. A thermocouple was sometimes used to enable the mean temperature of the specimen to be continuously observed during the ultrasonic treatment, and the temperature of the material could thus be controlled to within pre-set limits. Usually, however, it was undesirable to employ a thermocouple embedded in the living biological material, and in these cases the temperature control was investigated by means of dummy experiments carried out with similar specimens. The treatments were then made under identical conditions without the thermocouple. The brass cylinder G (Text-fig. 1) was electrically earthed, and contained water to a depth of 1 to 2 cm above a thin cellophane membrane. This membrane not only gave protection to the crystal generator, but the water layer it supported eliminated any possibility of the specimen becoming contaminated with transformer oil, which would otherwise have wetted the outside of the treatment vessel.

The general procedure was to treat the living material with ultrasonics, to allow it to continue to grow after treatment, and to investigate the course of the subsequent mitosis by means of permanent preparations of specimens fixed at regular intervals after treatment. The experiments were thus designed to show any similarity which might exist between the cytological effects of ultrasonics and those of ionising radiations or certain chemical agents. In all experiments a comparable number of control slides were prepared from material which had been handled in an exactly similar manner to the treated material, even to placing the material in the treatment vessel, but with the exception that no ultrasonic radiation was actually applied. Any observed effect of the ultrasonics could then be established by comparison with these controls.

### A. STUDIES WITH ROOT TIPS OF *Allium cepa*

The root tips of *Allium cepa* provide a source of plant cells in mitosis eminently suitable for cytological study. As the chromosomes are of similar length, any broken chromosome fragments resulting from an experimental treatment would be readily recognised as such. Seeds were allowed to germinate on damp filter paper in petri dishes in a constant-temperature box maintained at 20° C. Preliminary work had shown that it was necessary to avoid using the shortest seedlings for experimental work, as the first mitosis in such material is abnormal. Accordingly only roots from 1.5 to 2 cm in length were selected. No abnormal mitosis occurs in untreated roots of this lenght.

The seedlings were placed in water at the bottom of the treatment vessel (Fig. 1), and were held in position with a small piece of cellophane so that the root tips lay across the centre of the ultrasonic beam. Preliminary experiments showed that the temperature of the seedlings was controlled to within 2° C. of room temperature, where intensities of less than 10 watts/cm$^2$ were used. After each treatment had been completed, the seedlings were replaced on damp filter paper in a separate petri dish in the temperature-control box. The seedlings were fixed in acetic alcohol at half-hourly intervals from 0–6 hours after treatment. Permanent preparations were made for microsopical examination by the "Feulgen Squash Method" as described by Darlington and La Cour (6) in their book "The Handling of Chromosomes".

*Root Tips Treated in a Water Medium.* Examination of slides prepared after ultrasonic treatments at intensities of less than 2–2.5 watts/cm$^2$ for periods of 5 to 10 minutes showed no effect on the cells or on the course of their subsequent mitosis. The mitotic figures observed were all normal, and no marked increase or decrease in the rate of mitosis was noted. Untreated and treated seedlings which had been allowed to continue growing were indistinguishable from each other immediately after the treatment and during the course of the next few days.

Seedlings which had been exposed to ultrasonic intensities greater than 2–2.5 watts/cm$^2$ were disrupted in a manner characteristic of the mechanical effects of cavitation on living tissue. After treatment the roots bore obvious signs of damage: they were limp, fragile, and had a translucent and gelatinous appearance. Slides prepared immediately after such treatments showed that the nuclei had been fragmented and the contents scattered over the adjacent tissue. It was possible to distinguish fragments of broken chromosomes amid

the general disruption. Treatments for one minute at 5 watts/cm$^2$ were sufficient to cause disruption and the seedlings were incapable of further growth. Intensities of 2.5 watts/cm$^2$ applied for 3 minutes did not disrupt the cells, but subsequently the nuclei underwent pycnosis and the seedling did not recover.

No chromosome fragments or abnormalities of any kind were observed in material which was capable of further growth after ultrasonic irradiation.

*Root Tips Treated in Sugar Solution.* When a seedling is placed in water, a pressure of the order 10 atmospheres exists within the cells due to the osmotic effect at the cell membrane, this pressure being supported by the cellulose walls of the cell. A travelling acoustic wave passing through the material causes a periodic series of pressure fluctuations to be superimposed on the constant hydrostatic pressure. A constant pressure of 10 atmospheres existing within the cells would effectively prevent any state of liquid tension occurring during the negative part of the ultrasonic pressure cycle. These were the conditions of the experiments described above. In the following experiments the effect of ultrasonics on the root tips was investigated when no such pressure existed within the cells. These experimental conditions were achieved by treating the seedlings in a sugar solution having an osmotic pressure of 20 atmospheres.

A dozen seedlings with roots between 1.5 and 2 cm in length were soaked in a solution of dextrose of an osmotic pressure of 20 atmospheres for 20 minutes, and then treated with ultrasonics for 10 minutes in the same medium. The osmotic pressure of the medium was then gradually brought back to zero by placing the seedlings successively for 8 minutes in each of a series of sugar solutions of an osmotic pressure of 15, 10 and 5 atmospheres respectively. After being washed in water for 5 minutes, the seedlings were replaced on damp filter paper and were fixed at half-hourly intervals during the next 6 hours. A second group of seedlings was similarly treated in the sugar solutions, but no ultrasonic radiation was applied: these were also fixed at intervals of from 0 to 6 hours after treatment. Preliminary experiments had shown that the sugar treatment alone did not kill the seedlings although the rate of elongation of the root after treatment was slightly retarded. Six control seedlings having received no special treatment were also fixed. Permanent stained preparations of the root tips were subsequently made by the Feulgen method. The whole procedure was repeated in a series of experiments with ultrasonic intensities of between 1 and 10 watts/cm$^2$.

The results of the experiments were as follows:— a) Treatments at ultrasonic intensities greater than 2.5 to 3 watts/cm$^2$ caused disruption and death of the tissue. b) After treatments at intensities less than 2 watts/cm$^2$ no abnorm-

TABLE I

The Frequency of Bridge Abnormalities in Root Tips of *Allium cepa* after Treatment at an Intensity of 2.5 Watts/cm² for 10 Minutes.

| Time between treatment and fixation | Numbers of bridges in each root tip | Average number of bridges per root tip |
|---|---|---|
| 0 to 1 hour ..... | 1, 2, 3 and 2 | 2 |
| 1.5 to 2 hours ..... | 4, 6, 5, 4 and 5 | 5 |
| 3 to 6 hours ..... | 1, 3, 4, 1 and 2 | 2 |

ality could be detected. c) Treatments at an intensity of 2.5 watts/cm² for 5 to 10 minutes resulted in the production of chromosome bridges at anaphase and telophase in the subsequent mitosis (Plate 1A, B and C).

These abnormalities were observed in root tips together with cells and mitoses of normal appearance. Table I shows the number of bridges in each of 14 root tips which had received this treatment. It will be noticed that most bridges were observed in specimens fixed about 2 hours after treatment, and the bridge abnormality appeared to accumulate in the tissue up to this time. The total number of telophase figures in a single root tip preparation varied widely. The figure of 5 bridges observed per root tip represented a percentage abnormality at telophase of between 50 and 90 per cent. The uncertainty here arose from the fact that whereas a late telophase with a bridge is easily observed and counted, a normal late telophase develops into two resting nuclei and can no longer be identified. The bridges were not like those caused by ionising radiations in that no acentric chromosomes or chromosome fragments were ever observed in association with them. The bridges appeared to arise from the failure of the chromatids to separate at anaphase (cf. Fig 3 with a normal anaphase shown in Fig. 4). Bridges observed from 3 to 6 hours after treatment often appeared as filaments of chromatin stretched between two nuclei which had returned to a resting stage. The chromatin thread was usually very thin at this time and often difficult to observe: it may well have ruptured or dissolved, which would account for the decrease in the number of bridges counted in roots fixed more than 2 hours after treatment. The bridges belong to that class of cytological abnormality known as sticky chromosomes, cf. D'Amato (3). The preparations fixed from 3 to 6 hours after treatment also contained a number of pycnotic nuclei scattered in tissue which was otherwise unchanged and showed normal mitotic figures.

The root tips which had received no ultrasonic treatment, but had been

treated in sugar solution only, showed similar bridges. In twenty preparations there was an average of one abnormal bridge per root tip; this represents a percentage abnormality of between 5 and 20 per cent.

The corresponding control slides, prepared after no special treatment, showed one bridge in fifteen root tips systematically examined, which represents a proportion of abnormality of less than 1 per cent.

Thus the ultrasonic treatment of root tips of *Allium cepa* at intensities of 2.5 watts/cm$^2$, in a sugar solution medium, gave rise to bridges due to sticky chromosomes in the subsequent mitosis. The sugar treatment alone caused a similar effect but to a lesser degree. No chromosome fragments were observed in living material after treatment, and the metaphase figures appeared normal.

*Experiment Under Cavitation-Free Conditions.* Confirmation that the cellular disruption commonly observed with ultrasonic intensities greater than 2.5 watts/cm$^2$ is due to cavitation, was obtained in an experiment in which onion seedlings were treated in water under a hydrostatic pressure of 10 atmospheres. The experiment was performed jointly with Dr. M. H. F. Wilkins, with an apparatus in which the seedlings were placed within a plastic tube through which cooling water flowed under pressure. The root tips were treated for 10 minutes at an estimated intensity of between 3 and 5 watts/cm$^2$, allowance having been made for the ultrasonic absorption in the plastic. No cavitation was possible in the water surrounding the root tips, for the 10 atmosphere steady pressure prevented any state of liquid tension arising from the ultrasonic pressure fluctuations, and no cavities could be formed. Temperature control was to within 1° C.

The root tip preparations subsequently made were quite normal and contained only normal mitoses; there was no disruption or cell damage.

## B. EXPERIMENTS WITH CHICK HEART FIBROBLASTS IN TISSUE CULTURE

Chick heart fibroblasts grown in tissue culture provide a convenient source of avian tissue in active mitosis. The cells which have migrated from the explant form a thin layer, which is at once suitable for experimental treatment under controlled conditions and for subsequent microscopical examination. In these experiments the thin cell layer allowed the temperature of the tissue to be controlled to within 1–2° C. of the temperature of the Tyrode medium

in contact with the cells, and ensured that the ultrasonic intensities measured were those to which all the cells were exposed.

The material (*Gallus domesticus*) possesses 78 chromosomes according to Yamashina (25), including 12 which are larger than the rest, the smallest being 0.2 μ in diameter. Clearly the material is not ideal for the observation of chromosome fragments induced as a result of an experimental treatment. Fragments might go unnoticed unless the larger chromosomes were markedly affected or acentric fragments were left in the equatorial region at anaphase. The chromosomes are also prone to lag at anaphase before rejoining their respective groups. An occasional lagging chromosome was therefore classed as normal in this work, which often implied that a chromosome bridge could not be considered as a definite abnormality unless telophase had been reached.

Tissue fragments from the heart of 11–13 day chick embroys were explanted, by Dr. J. Marchant, in hanging drop cultures on No. 0, 1.5 cm. circular cover-slips attached by a drop of Tyrode to the underside of No. 2, 1.25 in. square cover-slips which were sealed with molten paraffin wax to 3 × 1.5 in. hollow ground slides. The culture medium consisted of 1 drop of fowl plasma mixed with 1 drop of 10—12 day chick embryo extract. The cultures were incubated at 37–38° C., and used for experiments on the second day of growth. A 1.5 cm. cover-slip was then unwaxed and placed in the bottom of the ultrasonic treatment vessel (Text-fig. 1), in a few c.c. of warm Tyrode, so that the culture faced the generator. The cells were in contact with Tyrode only, the cover-slip being raised from the bottom cellophane window of the treatment vessel by a thin wire ring of aluminium.

After ultrasonic treatment, the cover-slip bearing the culture was re-attached to its 1.25 in. square, which was rewaxed to the well slide. The whole was then replaced in the incubator. If a five minute ultrasonic treatment was being given, the entire operation from the time when the specimen was taken from the incubator to the moment when it was replaced, occupied 7 minutes. During the time that the culture was outside the incubator, its temperature might drop to about 20° C. The control slides, which were treated identically except that no ultrasonic treatment was given, showed that this momentary temperature drop had no effect upon the fibroblasts or the course of their mitosis. After a measured interval of time in the incubator following treatment, the cells were fixed in Maximow's fluid for a few minutes, after being rinsed in warm Tyrode. The cultures were then washed for some hours in running tap-water, hydrolysed as for the Feulgen method, and stained in Ehrlich's haematoxylin, cf. Hughes and Fell (10). In addition a few cultures were prepared by the Feulgen method after fixation in Susa's fluid; most preparations, however, were made by the technique as described above, which was preferred to the Feulgen method since it rendered the cytoplasm more distinct. In this way cultures were treated at a series of ultrasonic intensities, and the subsequent mitosis was observed at 15 minute intervals during the following 2 hours. Equal numbers of control slides were simultaneously prepared.

The results of the experiments were as follows: — a) Treatment at ultrasonic intensities greater than 2.5 watts/cm² disrupted and killed the tissue. b) Ultrasonic intensities less than 2 watts/cm² had no effect upon the appearance of the cells or the course of their mitosis. c) Ultrasonic treatments at an intensity of 2.5 watts/cm² for 5 minutes gave rise to sticky chromosome bridges. The bridges were observed in cultures which had not been dis-

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## TEXT TO FIGURES

Figs. 1 and 2. Squash preparations of *Allium cepa* showing sticky chromosome bridges at telo phase, fixed 90 minutes after treatment with ultrasonics of intensity 2.5 watts/cm² for 5 minutes in a sugar solution medium.

Fixed in acetic alcohol and stained by the Feulgen method. Magnification ×1 500.

Fig. 3. Squash preparation of root tip of *Allium cepa* showing bridges at anaphase caused by the failure of the chromatids to disjoin. Fixed 90 minutes after treatment with ultrasonics at 2.5 watts/cm² for 5 minutes in a sugar solution medium.

Fixed in acetic alcohol and stained by the Feulgen method. Magnification ×1 500.

Fig. 4. Squash preparation of root tip of *Allium cepa* showing a normal anaphase. No ultrasonic treatment. (Cf. Fig. 3.)

Fixed in acetic alcohol and stained by the Feulgen method. Magnification ×1 500.

Fig. 5. A composite photograph of two adjacent telophase figures showing bridges with marked chromatin agglutination in a tissue culture of chick fibroblasts, fixed 80 minutes after treatment with ultrasonics at 2.5 watts/cm² for 5 minutes in a Tyrode medium.

Fixed in Maximow's fluid and stained in Ehrlich's haematoxylin. Magnification ×2 400.

Fig. 6. Late telophase bridge showing chromatin agglutination in a tissue culture of chick fibroblasts; a thread of chromatin joins the two reforming daughter nuclei. Fixed 80 minutes after treatment with ultrasonics at 2.5 watts/cm² for 5 minutes.

Fixed in Maximow's fluid and stained in Ehrlich's haematoxylin. Magnification ×2 100.

Fig. 7. Abnormal early anaphase showing chromatin agglutination in a tissue culture of chick fibroblasts. Fixed 30 minutes after treatment with ultrasonics at 2.5 watts/cm² for 5 minutes.

Fixed in Maximow's fluid and stained with Ehrlich's haematoxylin. Magnification ×1 500.

Fig. 8. Sticky metaphase figure showing marked clumping of chromosomes in a tissue culture of chick fibroblasts; a small group of chromosomes is displaced from the metaphase plate. Fixed 60 minutes after treatment with ultrasonics at 2.5 watts/cm² for 5 minutes.

Fixed in Maximow's fluid and stained in Ehrlich's haematoxylin. Magnification ×1 900.

Figs. 9, 10, 11, and 12. Progressive stages of nuclear degeneration leading to pycnosis in tissue culture of chick fibroblasts. 9 shows a resting stage nucleus of normal appearance. At 10 the fine fibrillar network of chromatin has condensed to a coarse structure consisting of thicker fibres and coarser granules. At 11 the granules tend to adhere to the nuclear membrane so that the nucleus at 12 appears more sharply defined. Photographs 9, 10, 11 and 12 are of nuclei from cultures fixed 30-60 minutes after treatment with ultrasonics at 2.5 watts/cm² for 5 minutes; only a small proportion of resting-stage nuclei were of abnormal appearance.

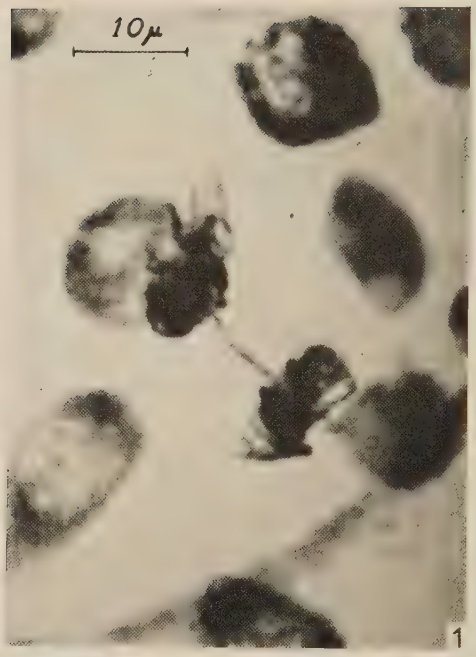
Fixed in Maximow's fluid and stained in Ehrlich's haematoxylin. Magnification ×2 300.

Fig. 13. Marked chromatin agglutination in a pollen tube of diploid *Tradescantia* grown on a medium without colchicine. Fixed 24 hours after treatment with ultrasonics at 2.5 watts/cm² for 5 minutes during germination.

Fixed in acetic alcohol, stained lightly with aceto carmine and strongly by the Feulgen method. Magnification ×1 100.

Fig. 14. Metaphase chromosomes in a pollen tube of diploid *Tradescantia* showing chromosome stickiness by their diffuse outline. The culture was grown on a medium without colchicine and fixed 24 hours after treatment with ultrasonics at 2.5 watts/cm² for 5 minutes during germination.

Fixed in acetic alcohol and stained by the Feulgen method. Magnification ×900.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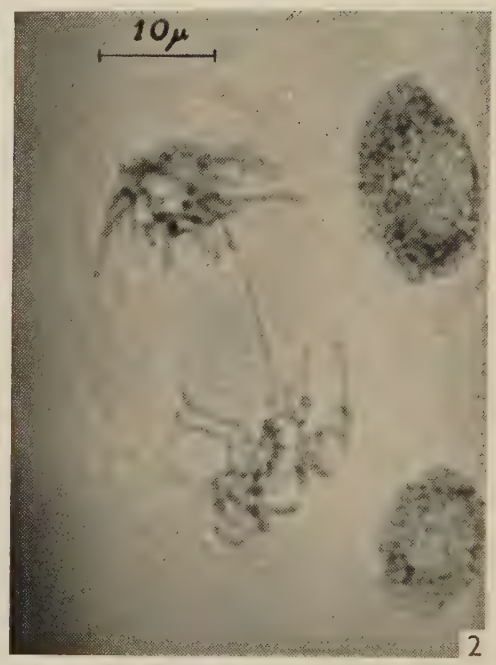
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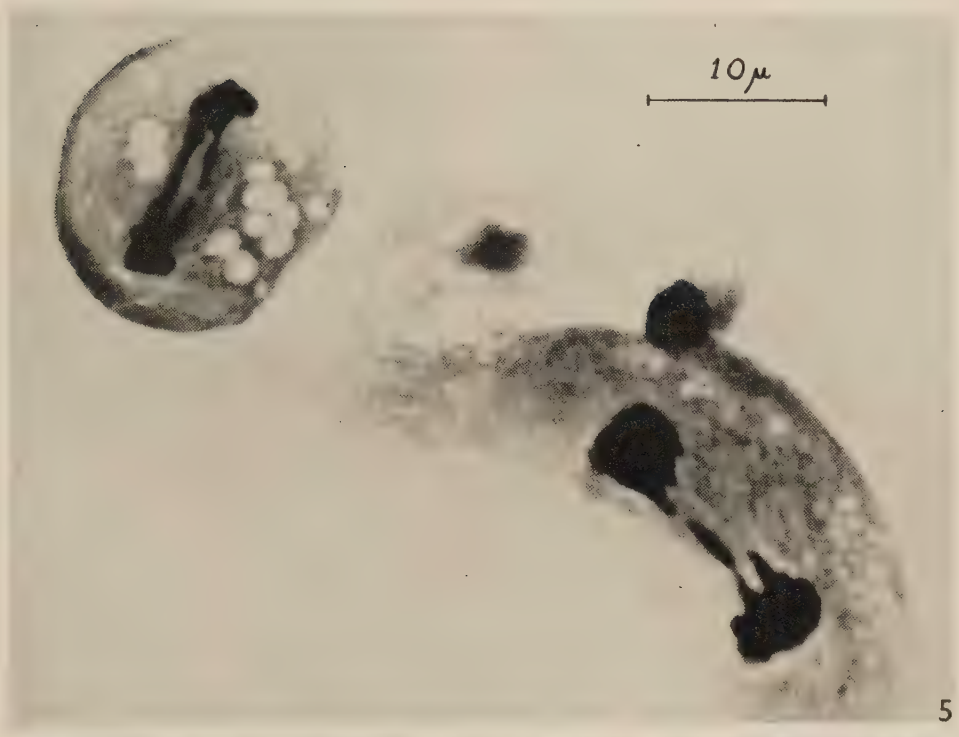


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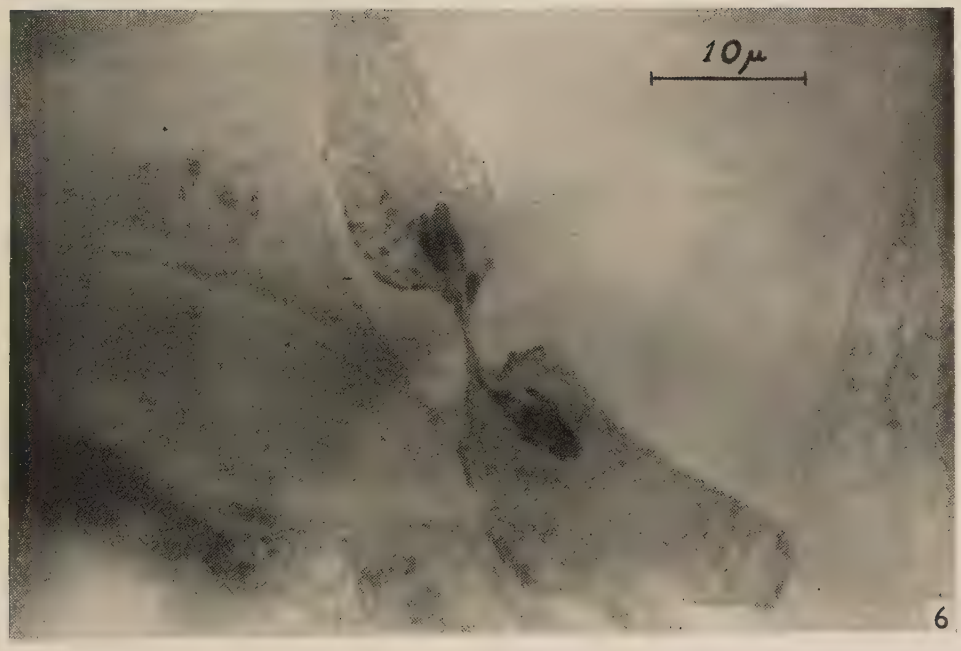


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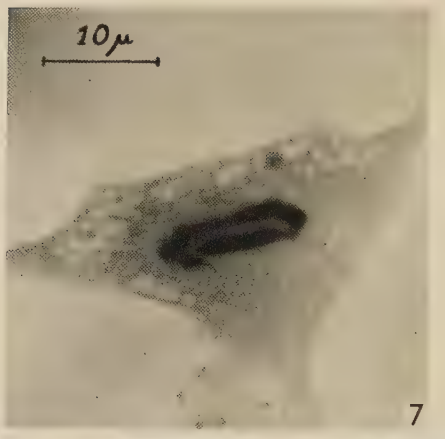
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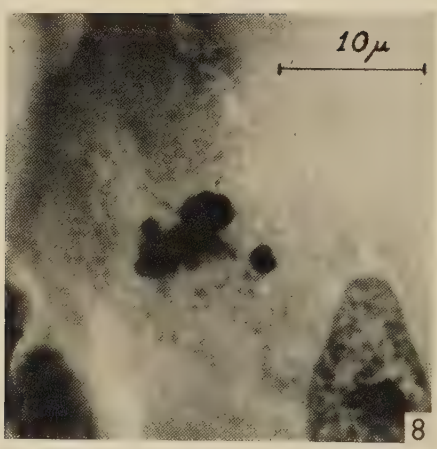
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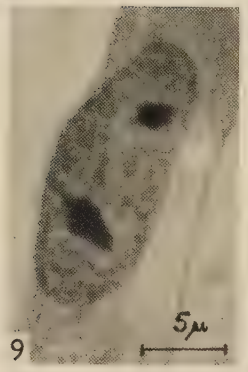
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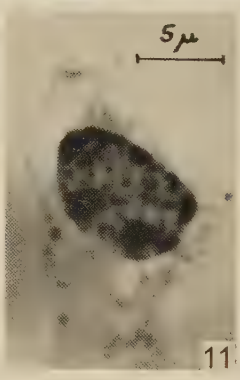
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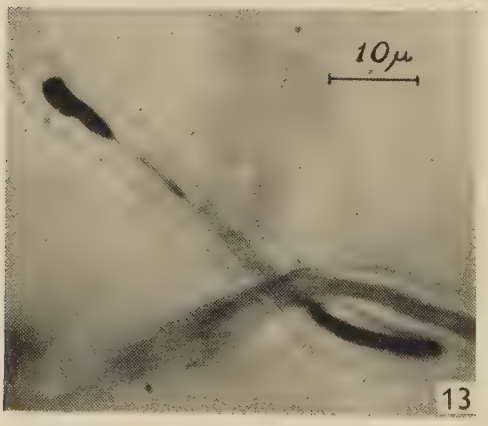
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rupted by cavitation and which also showed normal mitoses. In 11 cultures which had been fixed at times between 5 and 110 minutes after treatment 32 abnormal bridges in telophase or late anaphase were counted. The same slides also showed 60 normal telophase and late anaphase figures, giving an average percentage abnormality of approximately 35 per cent. One slide of a culture fixed 80 minutes after treatment contained 6 sticky chromosome bridges and only one normal telophase figure. This represents the maximum proportion of abnormalities seen in a single culture. In general the number of abnormalities observed in individual cultures after this treatment varied widely. About 40 control cultures were carefully examined, including 31 which had been placed in the treatment vessel; no bridge was encountered in any control slide.

The sticky chromosome effect was very pronounced: the chromosomes appeared to have fused together to give an appearance of chromatin agglutination. At telophase, bridges of chromatin were stretched between the reforming daughter nuclei. At metaphase and early anaphase the chromosomes were often clumped together, and individual chromosomes could not then be distinguished from each other. Occasionally a small group of chromosomes was displaced from the metaphase plate.

Most resting-stage nuclei in cultures fixed 30 -120 minutes after treatment at an intensity of 2.5 watts/cm$^2$ appeared normal. Individual nuclei could be found, however, which showed degenerative changes leading to pycnosis. The fine fibrillar chromatin network of the normal nucleus was replaced by a coarser structure in which both fibres and granules appeared to have coalesced to give thicker fibres and coarser granules. The latter tended to adhere to the nuclear membrane, and as a result the outline of the nucleus became more sharply defined. The nucleus probably shrank during these changes, which are similar to those which have been described by Glücksmann (9) for tissue which has been subjected to ionising radiation.

*Experiment with a Whole Chick Heart.* The heart of a 12 day chick embryo was cut into four parts. Three fragments were treated with ultrasonics at intensities of 2.5, 5 and 11 watts/cm$^2$ respectively, in a Tyrode medium in a sterilised treatment vessel. The temperature of the tissue did not rise above 30° C. during the treatment. The fourth part received no ultrasonics, and was used as a control. Each heart quarter was then divided into 12 approximately equal fragments which were explanted in hanging drop cultures. Four cultures from each treatment and the control were fixed in Maximow's fluid 19 hr., 29 hr., and 40 hr. after treatment, and were stained in Ehrlich's haematoxylin. The preparations were then examined for bridge abnormalities, which were counted together with the number of normal telophase and late anaphase figures.

TABLE II

Experiment with a chick heart: Counts of Bridges, Normal Telophase and Normal Late Anaphase Figures.

| Intensity of Ultrasonics (for 5 min.) | After 19 hrs. | | After 29 hrs. | |
|---|---|---|---|---|
| | Normal Divs. | Bridges | Normal Divs. | Bridges |
| 2.5 watts/cm$^2$..... | 3 | 0 | 12 | 0 |
| 5 watts/cm$^2$..... | 9 | 0 | 19 | 4 |
| 11 watts/cm$^2$..... | 0 | 0 | 3 | 1 |
| — Control ..... | 8 | 0 | 25 | 0 |

No detailed count of the mitotic figures was made for cultures of 40 hr. growth. It was observed, however, that the total number of bridges in the cultures was in no case greater than for the 29 hr. cultures, while the number of normal mitoses was considerably greater.

The results are shown in Table II. After treatment at 5 watts/cm$^2$, four sticky chromosome bridges were present in tissue which contained a majority of normal mitoses. After treatment at 11 watt/cm$^2$ very little growth occurred, and the cells exhibited marked vacuolisation of the cytoplasm. After all treatments some areas of the explant failed to grow, so that the zone of outgrowth was irregular. No chromosome fragments were observed.

In this experiment the tissue of the chick heart was of appreciable thickness when treated, and owing to the ultrasonic absorption in the tissue, all parts were not exposed to the same ultrasonic intensity. It is suggested that growth after treatments at intensities of 5 and 11 watts/cm$^2$ occurred from regions which had been to some extent sheltered by layers of cells nearer to the generator. The results provided some confirmation for the observations made in the previous experiments, while illustrating the superiority of the latter method of treating thin layers of cells in tissue culture.

### C. EXPERIMENTS WITH *Tradescantia* POLLEN TUBES

In pollen tubes of *Tradescantia* grown under artificial conditions, the generative nucleus divides into two sperm nuclei 12–24 hours after germination at room temperature. The six chromosomes are clearly visible at metaphase and are ideal for cytological studies. The effects of ionising radiations on this material are well established (22).

A hanging drop method of pollen tube cultivation was used in these experiments. The culture medium consisted of an 8 per cent solution of sucrose, 2 per cent agar

and 2 per cent gelatin, after Newcomer (14), freshly prepared in 25 c.c. lots with distilled water. The warm medium was applied in a thin smear to the centre of a No. 0, 1.5 cm circular cover-slip, over which the pollen of freshly-opened flowers of diploid *Tradescantia* was lightly dusted. The cover-slip was then inverted over a Van Teighen cell constructed from a glass ring attached to a slide with Canada balsam (1). The cell contained a drop of water at the bottom, and was sealed with a thin smear of vaseline. The whole was incubated at 19–20° C.

Direct microscopical examination of the pollen tubes during growth was possible provided that sudden temperature changes were avoided. Cytoplasmic streaming was used as evidence that the pollen tubes were still growing after treatment. The pollen tubes were treated upon their cover-slips in the same way as the tissue cultures. A 12 per cent aqueous solution of lactose was used as the treatment medium; preliminary experiments had shown that such a solution was of suitable osmotic pressure, and the use of lactose made easier the prevention of bacterial contamination. After treatment the cover-slip was carefully drained and replaced over the Van Teighen cell. Control experiments showed that the pollen tubes at any stage in their development could be placed for 10 minutes in the treatment vessel containing 12 per cent lactose, without their growth being affected. After growth, the specimens were fixed in acetic alcohol and stained by the Feulgen method. Where observations other than on the chromosomes were required, the pollen tubes were also lightly stained with aceto-carmine immediately after fixation.

For many experiments the culture medium contained 2 per cent colchicine. Colchicine arrests mitosis by inhibiting the development of the spindle with the result that in the present experiment metaphase was prolonged and metaphase chromosomes accumulated in the pollen tubes. Forty metaphase figures were sometimes visible in a typical slide after 24 hours' growth on a medium containing colchicine.

Pollen grains were treated with ultrasonics during germination. Treatment at intensities less than 2.5 watts/cm$^2$ for 5 min. did not prevent germination. Treatments at 3.5 and 5 watts/cm$^2$ for 3 min. destroyed the majority of the pollen grains, but a few of the survivors germinated. The pollen tubes were allowed to grow for 24 hours after treatment, the medium containing colchicine, and the material was then fixed and stained for examination. All the metaphase chromosomes appeared normal after the treatments. In cultures to which ultrasonic intensities of 2–2.5 watts/cm$^2$ had been applied, over 80 normal metaphase figures were counted, and no chromosome fragment or other abnormality was noted. The few individual pollen tubes which reached metaphase after treatments greater than 2.5 watts/cm$^2$ also showed chromosomes of normal appearance. In pollen treated 2 hours after germination, with an ultrasonic intensity of 2.5 watts/cm$^2$, 11 metaphase figures, all of normal appearance, were counted; other tubes did not reach metaphase or became detached from the culture medium during treatment.

The results again show no similarity between the effects of ultrasonics under these conditions, and the effects of ionising radiations.

An unsuccessful attempt was made to cause breakage of metaphase chromosomes within the pollen tubes. Three pollen tube cultures grown for 24 hours in a medium containing colchicine and therefore containing an accumulation of metaphase chromosomes, were treated at intensities of 2.5, 3.5 and 5 watts/cm$^2$ respectively for 3 minutes and were immediately fixed. After treatments at 3.5 and 5 watts/cm$^2$, much of the material was lost, but in a few cases breaks across the pollen tube itself were observed. No breaks in chromosomes within a pollen tube were seen, although after treatment at 2.5 watts/cm$^2$ about 10 undamaged metaphase figures were visible.

The experiments were repeated with no colchicine in the culture medium. Similar results were obtained although few metaphase figures were present after 24 hours' growth, as metaphase was no longer arrested. An interesting effect was noted here. It is normal in *Tradescantia* for the generative nucleus to precede the vegetative nucleus down the pollen tube. Examination of slides of grown pollen tubes showed that this occurred in all but 6 per cent of the tubes. After treatment at 2.5 watts/cm$^2$ during germination, however, the reverse order was noted in 41 per cent of the tubes (the counts being accurate to 4 per cent). Thus ultrasonic treatment had caused some disorganisation within the germinating pollen.

In the same experiments, the chromosomes in grown pollen tubes showed some signs of stickiness after treatments at 2.5 watts/cm$^2$ for 5 minutes during germination (Figs. 13 and 14). Stickiness was seen here in only four pollen tubes however.

## DISCUSSION

The experiments described in this paper show no evidence of any cytogenetical effects of ultrasonics. The experiments in which root tips of *Allium cepa* and pollen tubes of diploid *Tradescantia* were treated with ultrasonics and the subsequent mitosis was observed, were ideally suited for the observation of such effects. The chromosome complements in these materials are such that any fragmentation of chromosomes, such as that produced by ionising radiations in similar circumstances, would have been easily seen; no such fragmentation occurred, however. Mechanical stresses of sufficient magnitude to give rise to broken chromosomes within the cells could not be applied under the conditions of the present experiments without killing the tissue.

In a recent paper, Newcomer and Wallace (15) describe chromosome and nuclear aberrations induced in root tips of plant material by treatment with ultrasonics. The method employed by these workers was to expose root tips for a few seconds to intense beams of ultrasonics in water, and to fix them immediately after treatment. The photographs published show effects similar to those observed by the present author under similar conditions. The mechanically disruptive effects of cavitation are apparent and broken chromosome fragments are shown in metaphase figures. These immediate effects of ultrasonics are compared with the secondary effects of X-rays and mustard gas. The statement is made that: "The technique is genetically effective." In view of the fact that no attempt appears to have been made to allow the material to grow after treatment, or to investigate the course of the subsequent mitosis, and in view of the present author's experience that such a treatment invariably kills the cells, the genetical significance of the work seems doubtful.

The present experiments with chick heart fibroblasts show that bridges easily arise from sticky chromosome effects induced to a very marked degree in the mitosis following treatment with ultrasonics at an intensity of 2.5 watts/cm$^2$. The controls in these experiments were entirely free from abnormality. The corresponding experiments with *Allium cepa* were rather less conclusive in that controls which had been treated with sugar solution alone showed a rather similar effect though to a lesser degree. The conclusion that the ultrasonics produced additional chromosome stickiness was supported, however, by the results of the experiments with chick fibroblasts.

The sticky chromosome bridges are due to the failure of the chromatids to disjoin, and appear to have no genetical significance (21). Chromosome stickiness is usually seen after treatment with ionising radiations (2) or certain chemical agents such as mustard gas (10) or phenols (11). In these cases the stickiness is observed in conjunction with chromosome fragments and other abnormalities, but the chromosome stickiness induced by ultrasonics occurs in the absence of other effects. D'Amato (3) has studied chromosome stickiness in root tips of *Allium cepa*. Stickiness is induced by a number of inorganic solutions and by unfavourable environmental conditions such as water deficiency etc., but the precise manner in which the sticky chromosome effect arises in the nucleus is obscure. The hypothesis of Darlington (5), that stickiness is due to a surcharge of depolymerised thymonucleic acid, is widely accepted but is rejected by D'Amato (4). Pease (16, 17) describes experiments in which the sticky fusion bridges were induced by the application of external hydrostatic pressure of the order 3 000 lb/sq.

in., and suggests that his results may have been due to the reduction of gel rigidity within the nucleus. Extreme chromatin agglutination can be considered as due to the reduction of gel ridigity to the point of liquefaction. Moreover, cases of gel-sol transformations induced by ultrasonics are known (8).

In ultrasonic treatments of 2–2.5 watts/cm$^2$, destructive cavitation did not occur in contact with the tissue, or the tissue itself would have been disrupted. At this intensity, however, the possibility of some cavitation occurring in the surrounding aqueous medium cannot be disregarded. Chemical effects are known to be associated with cavitation induced by ultrasonics in water, and the cytological possibilities of this must be considered. On the basis of the chemical investigations of the author, it is estimated that no chemical change involving concentrations greater than 0.00003 mol/litre could occur in the water medium during this treatment. However, certain chemical agents in aqueous solution have been shown to have cytological effects at a concentration of 0.00001 mol/litre (11); thus the possibility that the chromosome stickiness described above was due to a chemical effect of ultrasonics cannot be excluded. The possibility is considered improbable, however, and cavitation not in contact with the cells but at some distant point in the water medium (adjacent to the cellophane membrane perhaps), would seem more likely to produce a direct mechanical effect due to shock fronts set up by the disturbance.

The possibility of producing cytological effects by means of intense beams of ultrasonics under cavitation-free conditions remains. Reports by Schmid and Rommel (19) and Mark (13) have claimed a depolymerising effect in high polymer solutions under these conditions, and the results were ascribed to a mechanism involving "viscous drag". However, recent investigations by Weissler (23) indicate that cavitation is necessary for such an effect to occur, and this author ascribes the results of Schmid and Mark to inadequate methods of eliminating cavitation. If Weissler's results are accepted, no work remains to illustrate the effects of the viscous drag mechanism, and it is probably wiser to try to establish such an effect on simple systems before biological experiments are attempted. The most favourable conditions for making such an experiment would be to use ultrasonics of wavelength of the same order of magnitude as the chromosomes themselves. This could be achieved by using ultrasonic frequencies of 50 Mc/sec. and above, but the ultrasonic absorption at these frequencies in biological material is so great that the problem of temperature control would be very difficult.

## SUMMARY

The effect of ultrasonic radiation on living tissue has been investigated with particular reference to mitosis. Observations were made on permanent preparations fixed at intervals after treatment.

Ultrasonic intensities of 2.5 watts/cm$^2$ applied to root tips of *Allium cepa* in a sugar solution medium of 20 atmosphere osmotic pressure gave rise to an increased number of sticky chromosome bridges in the subsequent mitosis. The cellular disruption produced at intensities greater than 2.5 watts/cm$^2$ was suppressed in an experiment using external hydrostatic pressure.

Ultrasonic intensities of 2.5 watts/cm$^2$ applied to chick fibroblasts in tissue culture produced chromatin agglutination and bridges during the subsequent mitosis; no bridge was encountered in any control slide.

Ultrasonic treatment of germinating pollen grains of *Tradescantia* affected the order in which the vegetative and generative nuclei proceeded down the pollen tube. Some chromosome stickiness was also induced in this material.

No evidence for any cytogenetical effects of ultrasonics was found: no chromosome breakage occurred as a result of ultrasonic radiation in tissue which survived treatment.

A temporary liquefaction of chromatin is suggested as an explanation of chromosome stickiness.


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# CYTOLOGICAL STUDY OF *THERMOBACTERIUM ACIDOPHILUS* R 26 CULTURED IN ABSENCE OF DEOXYRIBONUCLEOSIDES OR URACIL

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When the culture medium of a microorganism has become exhausted with respect to a component necessary for normal growth, the culture can continue to grow for some time. However, growth becomes less rapid and is no longer exponential. The intracellular concentration of constituents, the synthesis of which is most directly dependent on the absent component, diminishes owing to their "dilution" in an increasing number of cells. The observations are readily made on cultures of *Polytomella*, in which the intracellular concentration of ribonucleic acid is decreased when growth is limited by exhaustion of the phosphate ions of the culture medium (6). The study of the physiological characters of cells, thus impoverished in some constituent, might give indications as to its function.

We have used this method to study *Thermobacterium acidophilus* R 26, growth being limited by suppression from the medium of two essential components: either deoxyribonucleic acid (or deoxyribonucleosides) (5) or uracil, the first of these compounds presumably being necessary for the building of nucleoids, the second for that of cytoplasmic ribonucleoproteins. Our present results have been obtained by cytochemical methods. A quantitative analysis of these observations will be published at a later date.

## METHODS

Cultures of a strain maintained on skimmed milk containing cystein and yeast extract were grown in the medium used by Hoff-Jørgensen (5) for the microbiological assay of deoxyribonucleosides.

Constitution of the complete medium following Hoff-Jørgensen (slightly modified by us) per 100 ml.:

1.25 g HCl-hydrolyzed casein, 0.25 g pancreatin hydrolyzed casein, 1.5 g sodium acetate, 1.5 g glucose, 0.05 g tween 80, 0.01 g dl-Tryptophan, 0.1 g $KH_2PO_4$, 0.1 g $K_2HPO_4$, 0.01 g $MgSO_4 \cdot 7\ H_2O$, 0.002 g $MnSO_4 \cdot 4\ H_2O$, 0.001 g $FeSO_4 \cdot 7\ H_2O$,

1 mg adenine, 1 mg guanine, 1 mg thymine, 1 mg cytidine, 1 mg uracil, 0.05 mg riboflavin, 0.05 mg nicotinic acid, 0.05 mg calcium pantothenate, 0.05 mg p-Aminobenzoic acid, 0.005 mg pteroylglutamic acid, 2.7 mg deoxyribonucleic acid.

Seven ml of incomplete medium were seeded by inoculation with a small wire loop of culture in complete medium. Demonstration of nucleoids was accomplished, after hydrolysis in HCl, by Giemsa staining (Robinow). Feulgen staining was done after 10 minutes hydrolysis, controls consisting of non hydrolyzed cells. The ribonucleic acid content was estimated by differences between the quantity of fixed toluidine blue in ribonuclease treated and untreated cells (staining for 20 minutes in a saturated solution of dye, followed by 1 hour in ethanol for differentiation). Volumes of cells and nucleoids were determined by measurements on photomicrographs of the cells stained with Giemsa, before and after treatment by HCl at 60°.

## RESULTS

a) *Limitation of growth by decrease of the deoxyribonucleic acid (DNA) concentration of the medium.* The smaller the initial amount of DNA in the growth medium, the more both growth rate and maximal growth were decreased. With initial DNA concentrations of 1.5 μg/ml, abnormally long cells were visible; these became more and more numerous as the initial concentration of DNA was lowered. At 0.25 μg/ml DNA, or less, all the cells were elongated after a very slight growth of the culture. This was observed very regularly in more than 20 experiments, but never when the DNA concentration was normal.

Robinow staining showed numerous and widely spaced nucleoids in each cell, between which the cytoplasm was continuous. The cytoplasmic volume corresponding to each nucleoid was 35 to 40 times greater in the elongated cells than in normal cells, the volume of each nucleoid being approximately the same in both types of cells.

Cytological observations of elongated cells seemed to indicate that the cytoplasmic and nuclear constituents were normal, aside from their relative proportions. Cytoplasm as well as nucleoids stained as deeply with Giemsa as do normal cells; the same was true for toluidine blue staining. After ribonuclease action, toluidine blue staining of cytoplasm totally disappeared, that of nucleoids was extremely weak in all cases. Thus both cytoplasm and nucleoids contained considerable amounts of ribonucleic acid (RNA).

The Millon reaction did not give sufficient intensity to compare cytoplasmic protein content of both types of cells. However the high RNA content of elongated cells, their staining with Giemsa and fuchsin made it likely that increase in volume of elongated cells was due to synthesis of new cytoplasmic material.

The Feulgen reaction was positive but weak in the nucleoids of normal cells and was not detected in those of elongated cells.

b) *Reversibility of effects due to DNA exhaustion of the medium.* That elongated cells are not degenerate forms, physiologically deficient and permanently incapable of reproduction, was clearly demonstrated by the rapidity with which effects of DNA exhaustion could be reversed. Within 3 hours after normal amounts of DNA (27 μg/ml) have been added to a culture of elongated cells, a rapid increase in the number of the nucleoids was seen along the full length of each cell, although it was impossible to observe any division of existing nucleoids. The normal ratio between cytoplasmic and nucleoid volume was thus restored and the elongated cells then divided in series of segments containing only two nucleoids. Thus they assumed their normal appearance and their subsequent growth and multiplication was normal.

c) *Limitation of growth by decrease of uracil concentration of the medium.* In order to show that the effects of decreased DNA concentration of the medium were not the result of unspecific processes accompanying any inhibition of growth, we studied the cultures obtained in the absence of uracil.

Here the growth was slightly greater than in the absence of DNA, but stopped at optical density values far lower than when the medium was complete (0.04 instead of 0.22). In contrast to the observation on cultures made in low DNA media, cells whose growth has been interrupted by the absence of uracil were morphologically as well as cytochemically very similar to cells cultured in normal conditions. Two nucleoids could be seen in each cell and the ratio of cytoplasmic to nucleoid volume remained normal, although a very slight increase in cell volume was apparent.

Inhibition of growth in the absence of uracil, like that in the absence of DNA, was completely reversible.

## DISCUSSION

Microbiological literature contains many descriptions of bacterial or yeast cells becoming considerably elongated, but not dividing under the influence of various agents. Reviews have been published by Hinshelwood (3) and Nickerson (7). Usually elongation has been observed after treatment by agents such as proflavin, cresol, penicillin and cobalt. In some instances,

nucleoids have been demonstrated in elongated cells (1.7). Usually nucleoids are numerous and the ratio between nucleoids and cytoplasmic volume does not appear to be altered (i.e. under the action of penicillin (1)). Yeast cells where elongation was not accompanied by nuclear division have however been observed by Nickerson.

Caldwell and Hinshelwood (2), comparing elongated cells of *B. lactis aerogenes* obtained by action of m-cresol and proflavin, concluded that the DNA content of each cell per unit length was normal with m-cresol and very low with proflavin. Thus growth of a cell and multiplication of its nucleoids are dependent on mechanisms which can be separately inhibited by adequate treatments.

The present data entirely support this view and offer some indication as to the nature of the cellular constituents involved.

Let us consider cells of *Th. acidophilus*, which when grown in absence of DNA, become elongated but do not acquire the normal number of nucleoids corresponding to cytoplasmic volume. The absence of deoxyribonucleosides, indispensable for normal growth in these bacteria, prevents in all likelihood the synthesis of DNA and the formation of new nucleoids. However RNA synthesis continues, and it is not surprising to see that growth is not stopped if one remembers the probable relationships between protein synthesis and RNA (Brachet, Caspersson).

Let us consider, on the other hand, a culture of the same bacteria where growth has been inhibited by the absence of uracil in the medium. Uracil is very probably indispensable for RNA synthesis, the only cell constituent containing this pyrimidine base. If no new quantities of RNA are synthesized, growth inhibition will involve all proteins, including those of the nucleoids, and this inhibition of growth will not be accompanied by abnormal relative proportions of the principal cell constituents.

In conclusion, in the case of *Th. acidophilus*, we appear to be able to interfere selectively either with DNA or with RNA synthesis, a fact which may be very useful for the study of the physiological function, still very

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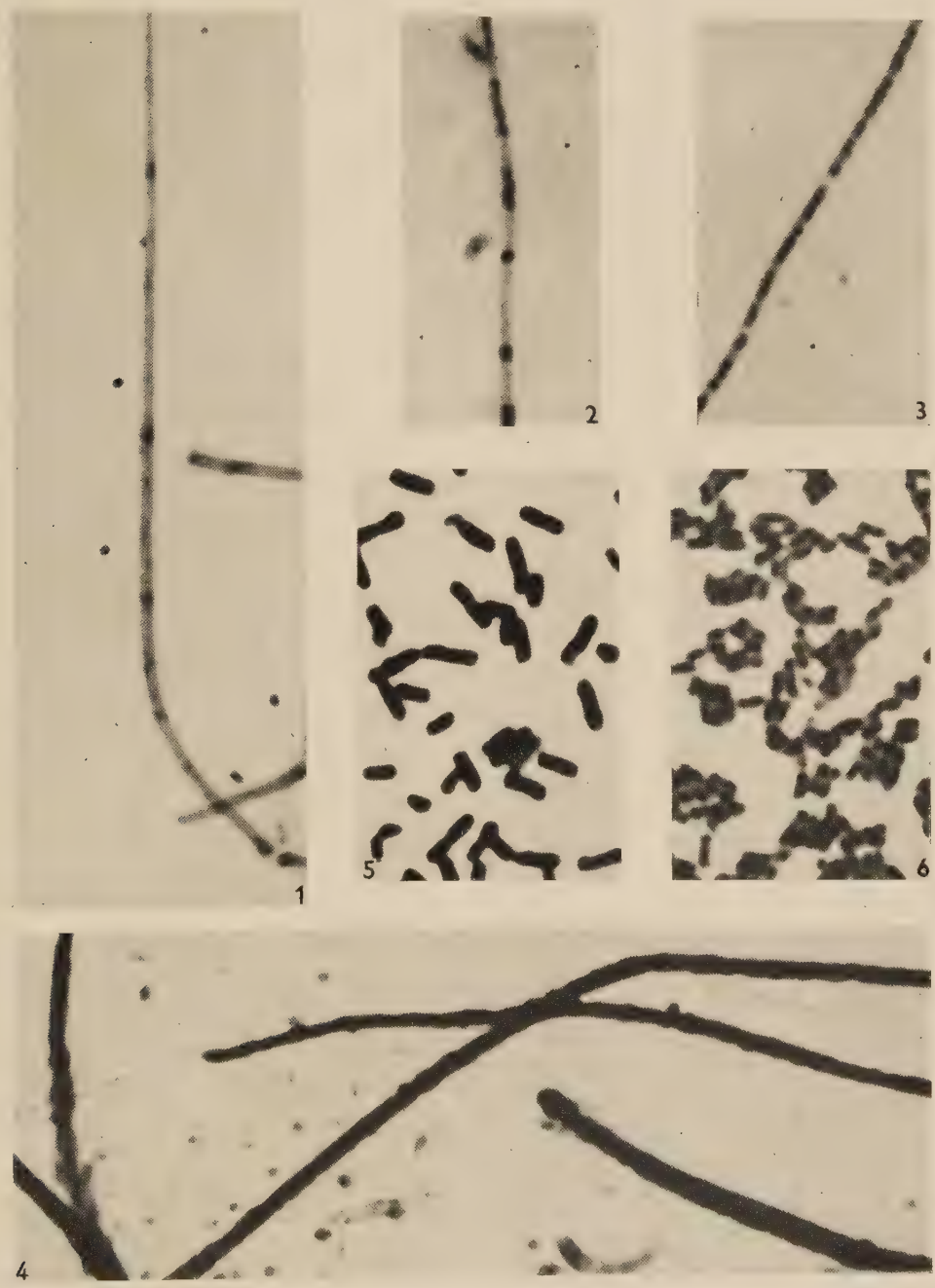
Fig. 1. *Thermobacterium acidophilus* R 26; cells cultured for 20 hours in DNA deficient medium; HCl-Giemsa. ×3400.
Fig. 2. *Th. acid.* R. 26; cells cultured for 20 hours in DNA deficient medium; HCl-Giemsa 4 hours after addition of 27 μg DNA per ml. ×3400.
Fig. 3. Same experiment as fig. 2; HCl-Giemsa 11 hours after addition of 27 μg DNA per ml. ×3400.
Fig. 4. Same experiment as fig. 1; Giemsa staining without previous action of HCl. ×3400.
Fig. 5. *Th. acid.* R 26; growth stopped by lack of uracil in the medium; Giemsa staining without previous action of HCl. ×3400.
Fig. 6. Same experiment as fig. 5; HCl-Giemsa. ×3400.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obscure, of both these substances. It seems that both are indispensable for indefinite growth and cellular multiplication. Furthermore, we might have a confirmation, as already apparent from their localization in different organites and their very different metabolic activity in higher organisms (i.e. P turnover in DNA and RNA), that both have distinct physiological functions. Indeed, our observations indicate that if protein synthesis is quantitatively related to that of RNA, its dependance on the presence of DNA is much more remote and indirect.

Finally, cell division is stopped before that of the nucleoids in cultures deprived of DNA; a minimum quantity of DNA per unit cell volume seems thus a necessary condition for division.

## SUMMARY

The ratio between the cytoplasmic and nucleoid volume is considerably increased in *Thermobacterium acidophilus* R 26 cells when growth rate is decreased by the lack of deoxyribonucleic acid in the culture medium. It remains constant when the limitation of growth is obtained by suppressing uracil, presumably indispensable for ribonucleic acid synthesis.

We are greatly indebted to our colleague J. Brachet for providing us with a strain of *Thermobacterium acidophilus* R 26 and a copy of an unpublished work by Hoff-Jørgensen on the microbiological essay of deoxyribonucleosides.

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# THE INTERPRETATION OF APPEARANCES IN THE CYTOLOGICAL STAINING OF BACTERIA

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Because of their small size, and because their reactions to standard staining and fixation techniques are often entirely different from those of plant and animal cells, the interpretation of cytological appearances in bacteria is an exceedingly difficult and specialised task, and frequently causes misconception and confusion.

In many bacteria the cytoplasm stains very deeply with "nuclear" dyes, and sometimes may cause the nuclear structures to appear lightly stained against a dark background, where they are not entirely concealed. The HCl hydrolysis technique of Piekarski (12) was introduced with the purpose of avoiding this difficulty, and has been employed with considerable success by many workers.

A second important point in the study of bacterial cytology is the difficulty of demonstrating the cell wall without the use of mordants. Many species of bacteria are subdivided into small cells by crosswalls derived from the cell wall, and these crosswalls are secreted by cytoplasmic septa containing an element which stains with basic dyes. Thus in preparations stained by simple cytological methods, the multicellular structure of the bacillus is not apparent, whereas the interpretation of the nuclear structures is complicated by material, of similar staining reaction, derived from the cell envelopes. This problem was well explained by Robinow (13), but many conscientious studies have since been rendered valueless because the authors, unaware of, or choosing to ignore the structure of the cell envelopes, have interpreted complex multicellular bacteria as single cells. This applies, for example, to the studies of Knaysi, Hillier and Fabricant (9) upon *Mycobacterium tuberculosis* and to a paper, which recently appeared in this Journal, by DeLamater and Mudd (5). In the latter paper, the authors interpreted various appearances in *Bacillus megatherium* as classical mitotic figures. They were, in the opinion of the present writer, caused by the effect of unsuitable fixation methods upon this organism, which DeLamater and Mudd apparently believed to consist of multinucleate cells, whereas, in fact, each of their

"nuclei" was a complete cell of this multicellular bacillus (Fig. 1). This question is further discussed below. Although it is, as a rule, exceedingly difficult to demonstrate both cell walls and nuclear structures simultaneously, the problem has been solved, for some bacteria, including *Bacillus*, by Cassel (4).

Nearly all fixation methods result in some degree of shrinkage and distortion of the bacterial nuclei and cell structures, and it is agreed by the majority of bacterial cytologists that they are best avoided. In a recent study, by the use of the electron microscope, Møller and Birch-Anderson (10) have shown that aqueous solutions of fixatives cause immediate shrinkage of the cell, whereas non-aqueous fixatives, although they do not cause obvious shrinkage, fail to protect the cell against such distortion when it is brought about subsequently by other causes; i.e., they do not "fix" at all. Thus fixatives are either dangerous or valueless. The shrinkage caused by drying or dehydration techniques need not be emphasised, and obviously it cannot be averted by prior recourse to fixatives. Plain water-mounts give excellent pictures with bacteria, and if they are examined immediately after they are prepared, no charge of mishandling of biological material can fairly be made.

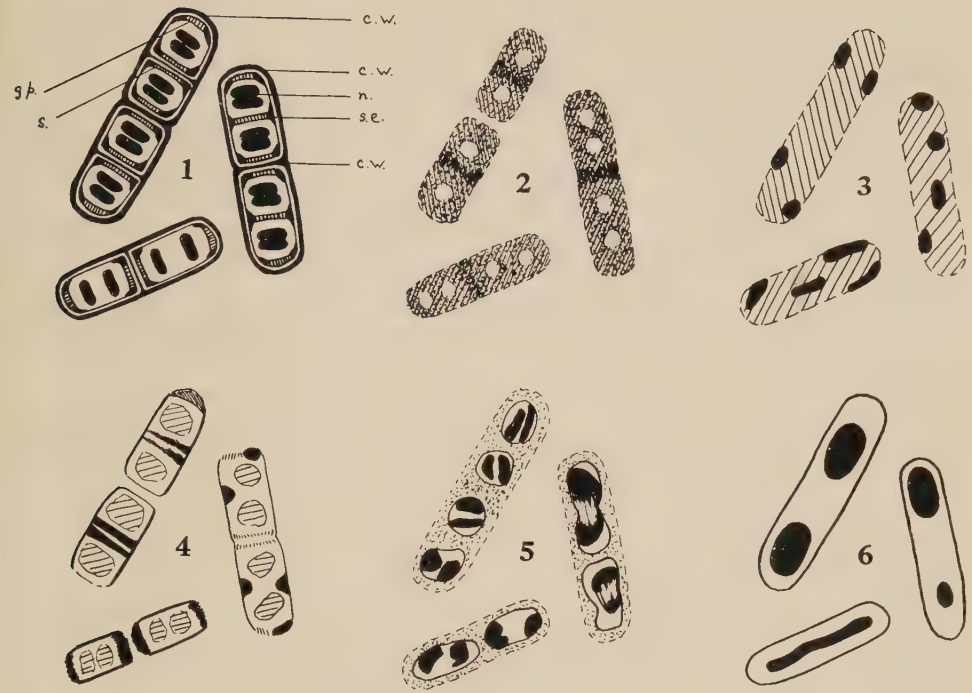
In this paper various appearances which are seen in cytological preparations of bacteria are described and their interpretation is discussed.

## APPEARANCES AND THEIR INTERPRETATIONS

As fuller descriptions of these staining techniques and of the morphology of bacteria are given in the author's monograph (1), the following account is confined mainly to microscopic appearances.

In Figs. 1 to 6 the same group of vegetative bacilli is shown as it appears after treatment by a variety of different techniques. The large, sporing bacilli of the genera *Bacillus* and *Clostridium* are usually of this type of morphology. The bacilli are in different stages of cell division, which are explained in the caption. In Fig. 1 the structure is seen in detail; there may be two or four cells in each bacillus, divided by protoplasmic septa, crosswalls or both. The nuclei are paired structures, short rods, dividing lengthwise like the chromosomes of higher organisms.

In Fig. 2, the same group is seen as it would appear when stained by a simple basic dye, such as methylene blue or thionin. The cell wall and crosswalls are not stained, but appear either as gaps or are indicated by the staining of the associated septa. The nuclei are seen as less deeply stained areas in the cytoplasm.




Figs. 1–6. A group of *Bacillus* fixed and stained by various methods.

Fig. 1. Diagram of the cytological structure of the organisms at various stages of vegetative cell division. The top-left bacillus is divided into four cells by complete cross-walls, and the cytoplasmic septa by which the walls are secreted. Fission is commencing at the centre cross-wall. The top-right bacillus is divided by one complete cross-wall and subdivided by cytoplasmic septa alone, in which new crosswalls have not yet commenced to form. The bottom-left bacillus has recently divided, and has only two cells.

c.w. — cell wall; g.p. — growing point; s. — cytoplasmic septum secreting crosswall; s.e. — cytoplasmic septum in early stage, before secretion of crosswall. (The darkly-stained corners of the septa are growing points, at the junctions of the septa and cell envelopes); n. — nucleus.

Fig. 2. The same group of bacilli seen as stained by a simple basic dye.

Fig. 3. As stained by Gram's stain and over-decolorised.

Figs. 4 and 5. Artefacts caused by fixation techniques, showing spurious mitotic figures, as in DeLamater and Mudd (5).

Fig. 6. Appearances seen in sporulating bacilli. (Explanations in the text.)

In Fig. 3, the appearance is that of preparations stained by the para-rosaniline-iodine Gram complex. The complex is readily removed from bacilli of this type in the process of differentiation, leaving granules of stain which, although associated with the surface structures of the bacillus, may give the appearance of internal structures.

In Figs. 4 and 5 are shown artefacts of a type which are frequently caused by unsuitable fixation techniques, such as the use of Bouin's solution,

formaldehyde, or alcohol, and sometimes by attempts to dehydrate and make permanent mounts of the cells by the methods employed for animal and plant tissues. The appearances in Fig. 4 are caused by selective staining of the basophile elements in the protoplasmic septa and growing points. These may appear as bars across the cell, or as granular pools of stain at the point of junction of cell wall and crosswall. The cells proper resemble vacuoles. The illustration of the cytological structure of a bacillus in the otherwise admirable work of Gautheret (6) shows an organism in this condition, and is accordingly misinterpreted, the septa being described as nuclei. In Fig. 5 the shrinkage caused by improper fixation has given the cells the appearance of nuclei within a unicellular bacillus.

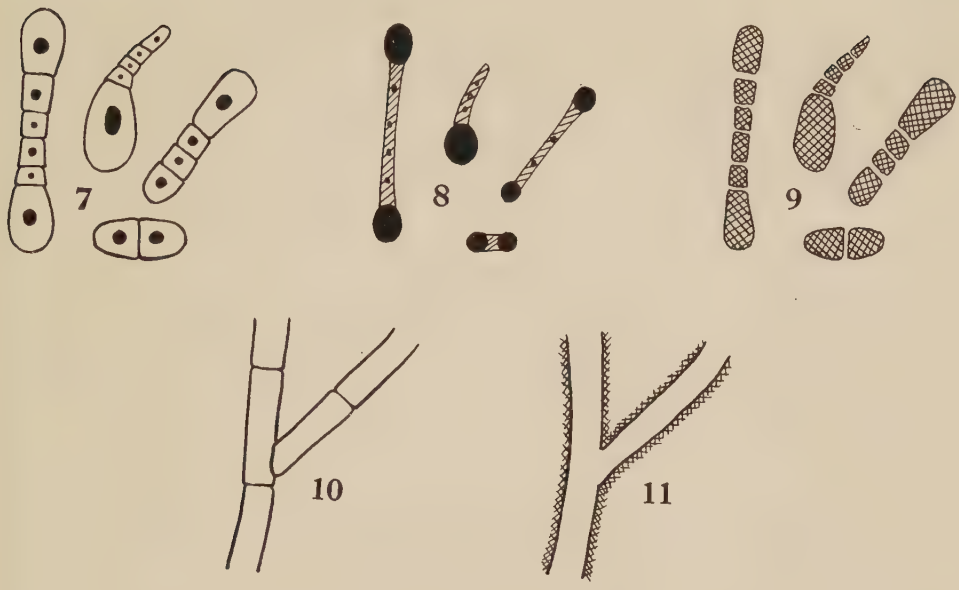
In the paper of DeLamater and Mudd (5), referred to above, the "mitotic figures" occurred in bacilli such as those in Figs. 4 and 5. The "interphase", "prophase", and "telophase" nuclei were entire, shrunken cells, as in Fig. 5. The "centrioles" were derived from the protoplasmic septa, as in the right-hand bacillus in Fig. 4.

In Fig. 6 a similar group of bacilli is shown with the appearances found in the process of sporulation. The cross-walls have broken down, and large bodies of chromatinic material appear in the positions of the incipient spores, or as longitudinal rods. These structures are perfectly genuine, but there always exists a danger that bacilli in this condition may be interpreted as vegetative cells, and the nuclear bodies incorrectly described in consequence (6). An excellent account of the cytology of sporulation is given by Klieneberger-Nobel (8).

The problem of the interpretation of cytological appearances in multicellular bacteria of a different type is explained in Figs 7 to 9. Fig. 7 shows a group of *Corynebacteria* or *Mycobacteria*, of which the morphology is similar. They are less regular in their morphology than *Bacillus*, and the terminal cells are often enlarged.

In Fig. 8 the same group is shown as it would appear when stained by one of the "diagnostic" stains employed for the identification of *C. diphtheriae* by medical bacteriologists. In this species the contents of the enlarged, terminal cells stain very deeply with suitable dye-complexes, and appear in heat-fixed preparations as the "metachromatic granules".

A somewhat different appearance is seen in Fig. 9, which represents the condition seen frequently in *M. tuberculosis*, when it is heat-fixed and stained. The cell contents have shrunken away from the unstained cell wall, and appear as a string of granules. In a recent study, Mudd *et al.* (11) have chosen to interpret these as "mitochondria".




Figs. 7–9. A group of *Corynebacterium* or *Mycobacterium*, showing true and "typical" appearances.

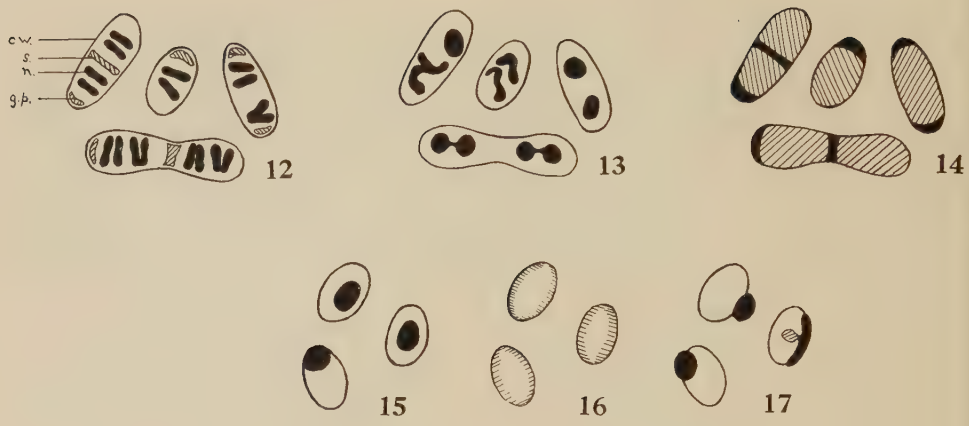
Fig. 7. Diagram of the cytological structure of these bacteria, showing irregular cell size, with enlarged terminal cells and small central nuclei.
Fig. 8. "Metachromatic granules," the shrunken contents of the terminal cells, by Neisser's stain or similar "diagnostic" procedures for *C. diphtheriae*.
Fig. 9. The beaded appearance seen in dried, heat-fixed preparations, especially of *M. tuberculosis*, stained by the Ziehl-Neelsen technique. The cell wall does not stain, and the granules are the shrunken cell contents.

Figs. 10–11. A temporary branch, characteristic of species of *Nocardia*, *Actinomyces*, *Mycobacterium* and occasionally *Corynebacterium*.

Fig. 10. The true structure, showing crosswalls, and the development of a wall between the mother cell and the branch.
Fig. 11. Inadequate mordanting of cell wall preparations causes the stain to accumulate on the surface, and gives a misleading picture of a true branch.

Bacteria of this type occasionally produce bud-like branches which readily become separated from the mother cell. Mordants, such as tannic acid, are employed to render the cell wall stainable; inadequate exposure to the mordant may cause the stain merely to accumulate upon the surface of the cell wall, concealing the crosswalls and giving the bud the appearance of a true branch (Figs. 10, 11).

The bacteria which have been the most frequent subject of cytological study, the *Bacteriaceae*, especially *Bact. coli*, do not have crosswalls, but are




Figs. 12–14. A group of *Bacterium*, indicating the basophilic structures.

Fig. 12. Diagram of the cytology of a *Bacterium*. The bacteria are unicellular and divide by constriction of the cell wall. Division is preceded by the formation of a cytoplasmic septum.

c.w. — cell wall; s. — cytoplasmic septum; g.p. — growing point (often at one end only); n. — nucleus.

Fig. 13. Artefacts in the appearance of the nuclei when heat-fixed, dehydrated or unsuitably fixed.

Fig. 14. The septa and growing points are the most obvious structures in cells lightly stained with aqueous basic dyes.

Figs. 15–17. Bacterial spores.

Fig. 15. The true position of the spore nucleus.

Fig. 16. Unstained refractive spores, as seen in unhydrolysed preparations.

Fig. 17. The effects of excessive acid-hydrolysis. In the two, lefthand spores the nuclear material has been ejected, and forms the "peripheral nucleus." In the right-hand spore, the nuclear material forms a pool between the cytoplasm and the spore coat, "the crescentic nucleus," as in Robinow (13). The original, central site of the nucleus is shown hatched.

unicellular and divide by constriction of the cell wall. In addition to the paired, rod-like nuclei or chromosomes, the growing points of the cells and the cytoplasmic septa at the points of cell division contain basophilic nucleic acids, as shown in Fig. 12. Often these cells grow only from one end (2).

In Fig. 13 are seen the effects of drying or improper fixation upon the nuclei of such cells. The rod-like chromosomes may shrink to a spherical granule or give the appearance of elongating to form an irregular filament of chromatin. The granular masses often approach one another, so as to give the appearance, frequently figured in early cytological work, of being attached by a narrow thread. This appearance is usually, but not always, an optical illusion.

If bacteria of this type are stained very briefly with aqueous basic dyes, the most obvious structures which appear are the growing points and cytoplasmic septa (Fig. 14). These have been confused with the nuclear structures by some observers (7), and with mitochondria (11).

Some of the descriptions of the morphology of bacterial spores which have been published have been based upon artefacts caused by acid-hydrolysis techniques. The author has recently published a full account of these effects (3), which need only briefly be described here. Fig. 15 shows the position of the nucleus in different types of spore. In Fig. 16 the spores are seen as they appear, unstained and refractive, when treated with simple dyes. In Fig. 17 they are shown in two of the conditions described by various workers, notably Robinow (13, 14), who originally favoured the concept of the "discrete, peripheral nucleus", as seen in the two left-hand spores; but who later decided that this was an artefact, and that the true nucleus took the form of a flat, crescentic surface structure (14). In my opinion (3) these appearances are both artefacts, and the spore nucleus, which can both be stained, and observed in the living spore by phase-contrast, is a central or slightly eccentric body, whose position is marked, in many of the hydrolysis artefacts, by a central body of lower staining capacity. The explanation of the ejection of the nuclear material, under the stress of acid-hydrolysis, is probably concerned with the concentration and turgour of the contents of the mature spore. Immature or germinating spores can be hydrolysed without ill-effect.

## SUMMARY AND CONCLUSIONS

1. Various appearances which may be found in the cytological staining of bacteria, and which may be the cause of confusion and error, are described and discussed.

2. All forms of fixation and dehydration are liable to cause distortion in bacterial cells, and their employment is deprecated.

3. Many types of bacteria are multicellular. The crosswalls which subdivide them are not easily demonstrated, and in such bacteria the entire cell units may be mistaken for nuclei. The interpretation of their cytology is further complicated by the occurrence of basophilic structures associated with the cell wall and crosswalls.

4. The misinterpretation of appearances of this type is, in the opinion of the author, responsible for reports of classical mitotic spindles in bacilli, extra cytoplasmic spore nuclei, mitochondria and other observations which are discussed in the text.

I wish to acknowledge the valuable assistance which has been given to me in the collection and correlation of the observations upon which this paper is based over, a period of six years, by Miss C. M. F. Hale.

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# HISTOCHEMICAL PREPARATION FOR AUTORADIOGRAPHY

## I. APPLICATION TO $BR^{82}$ [1]

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Among the numerous methods for histologic autoradiography advanced in recent years (1, 3, 7, 8, 9, 13, 17, 18, 19, 23), the strip-film method according to Pelc (7, 23) possesses the best resolving power hitherto obtained, viz. 2 to 3 microns (7, 15, 16, 20, 21, 23, 26, 27). It still seems doubtful whether particle track autoradiography can give better resolving power (6, 14, 15).

The strip-film method requires a thin histologic section ($\leq 5$ microns). The tissue has therefore usually been fixed and embedded in paraffin. A further condition is that the specimen passes through water before the film is applied.

For certain histochemical work ordinary fixation, embedding, and deparaffination can be sufficient (11, 16, 18). Evidently, such experiments are based on the assumption that the fixative precipitates the organically bound isotope without any diffusion during the time of the fixation. This may be the case, but has hardly been established. Nor is it known for certain whether some part of the organically bound isotope is liberated again in the ensuing water-bath.

In experiments to demonstrate autoradiographically inorganic or otherwise readily diffusible compounds of a certain isotope the preparation of the tissue is evidently the decisive factor. Scott Russell *et al.*, in preparing autoradiographs to show the distribution of $P^{32}$ in plant tissues, dealt largely with inorganic or easily diffusible phosphorous compounds (24, 25). For this reason they froze the tissue in isopentane at –70° C., transferred it to alcohol saturated with basic lead acetate maintained at –70° C., and finally embedded it in paraffin. They assumed that the ice in the tissue dissolved in the cold alcohol and that the phosphate was precipitated *in situ* as the lead salt. It is true that this hypothesis is borne out by the results, but there is hardly any proof that the phosphate is precipitated without diffusion.

A prerequisite of a more generally applicable method of tissue preparation for autoradiography is that it definitely eliminates the diffusion of the

[1] This study was aided by a grant from the Medical Faculty of the University of Lund.

Fig. 1.

Fig. 2.

Fig. 1. The slide holder with the movable diaphragm to the left.
Fig. 2. The slide holder in position under the G-M tube.

isotope under investigation throughout the entire preparative process from vital tissue to histologic section ($\leq 5$ microns in thickness) soaked in water. The present article reports such a method applied to $Br^{82}$ in inorganic compounds.

## METHODS AND APPARATUS

Freezing-drying according to the Altmann-Gersh principle was chosen as the first step (2, 22). A vacuum-drying apparatus, constructed by B. Gustavsson, Lund, and similar to the one described by Mendelow and Hamilton (22), was used in the present investigation. It differs from that of Mendelow and Hamilton in that it works at a moderate vacuum ($10^{-2}$ to $10^{-3}$ mm. Hg) with the tissue placed at a short distance from the cold wall. Complete dehydration of small pieces of tissue at about $-35°$ C. was obtained in 12 hours. A detailed description of the apparatus and its capabilities will be given elsewhere by Gustavsson (12).

With the aim of finding a method of preparing tissue which eliminates the liberation of the active compounds after drying, the activity of sections of the dried and paraffin-embedded tissue was measured with a G.M. tube when embedded in paraffin and again after every subsequent step of the histochemical treatment. In order to obtain an exact reproduction of the geometry on repeated measurements of the same slide, the latter was fixed in a special slide holder. This holder was provided with a base with a fixed position in relation to the G.M. tube and a movable slide table whose position

can be read on a scale with a vernier. Above the slide there is a diaphragm, by means of which a definite field of vision of a large histologic preparation is brought into focus (Figs. 1 and 2).

For measuring the comparatively low activities of β-radiation of the histologic sections, a small G.M. tube with a low background proved to be appropriate, viz. a self-quenching mica end-window tube, approximate volume 2.25 $cm^3$, window thickness 2.5 $mg/cm^2$ (Bruel and Kjaer, Copenhagen, type M1). Screened with 2.5 cm. lead the background was approximately 4 imp./min.

In the arrangement described the standard error of a single determination of activity was found to be low and practically independent of self-absorption and variations in geometry.

## EXPERIMENTS WITH $BR^{82}$

The biologic material employed in these experiments was part of earlier metabolism studies carried out by Brattgård and Lindqvist (4).

A cat weighing 1.850 kg. was given 2.8 mC $Br^{82}$ intravenously in the form of potassium bromide without carrier in physiological saline. The cat was killed 6 hours later by means of carbon dioxide. Bits of the cerebellum were plunged in chilled isopentane (–70° C), vacuum-dried for 18 hours in Gustavsson's apparatus at –25° C. and embedded directly in paraffin at +40° C. for 20 minutes. Due to the transportation of the paraffin blocks the treatment was discontinued for 13 hours.

Slides, treated with gelatine-chrome alum according to Doniach and Pelc (7), were smeared in the centra with a little albumin glycerin. On the sticky and practically dry surface sections of 5, 10, and 20 microns were mounted.

The activity of 7 sections (10 and 20 microns) was measured before and after 10 minutes' treatment with warm ether (as regards choice of ether, cf. Glick (10)). The result was an apparent gain in activity (mean +1.5 per cent) within the limits of the experimental error. The bromine was thus not dissolved in any measurable amount in the paraffin or in the ether-soluble tissue-fraction. Paraffin-embedding can therefore be assumed not to cause any diffusion of bromine in the tissue.

Experiments with 1 per cent silver nitrate in aqueous solution (together with some dioctyl ester of sodium sulfosuccinic acid, Aerosol OT, for rapid and complete wetting) produced no loss of activity, neither did ensuing baths in water — absolute alcohol — water. The treatment can therefore be assumed to cause precipitation of the bromine *in loco*.

Finally, attempts were made to precipitate proteinbound bromine fractions. A direct treatment of two deparaffinated sections with absolute alcohol and thereafter with water, gave 98.7 and 99.6 per cent loss in activity, respectively. Alcohol acidified with acetic acid (2 + 1), followed by water, produced a loss of 98.8 and 100.9 per cent. A preparation which was treated with 10 per cent trichloracetic acid followed by water, lost 50 per cent of its activity. It is not quite clear why a lower figure was obtained following treatment with trichloracetic acid than after alcohol fixation. Anyhow the results seem to indicate that the bromine was entirely non protein-bound. Experiments along the same lines but with other protein precipitants are in progress with a view of proving that the bromine is not bound to protein fractions.

Thinner sections (5 microns) of the frozen-dried paraffin-embedded cerebrum were therefore prepared for autoradiography as follows: 1. Warm ether for 10 minutes, divided into two baths. — 2. 1 per cent silver nitrate + some Aerosol OT in water for 5 minutes. — 3. Distilled water for 5 minutes. — 4. Absolute alcohol for 5 minutes. — 5. Distilled water for 15 minutes. — Autoradiography (exposure for 4.7 days), development, fixation, nuclear staining, and mounting in a glycerin-jelly medium according to Doniach and Pelc (7).

The autoradiographs displayed a diffuse granulation — much more marked than the background — throughout the whole cerebellum, in some spots with greater density over the cell bodies than interstitially (Figure 3). Sections from the same material and treated in the same way but not until the activity had had time to fall to 1 per cent of the initial value, gave no autoradiograms. The silver precipitation used thus did not influence the findings. A more comprehensive review of the biological results is published elsewhere (5).

## SUMMARY

A method for the preparation of tissue for autoradiography is described. Applied to $Br^{82}$ in inorganic compounds, it involves the following stages: Freeze-drying in a quick-working vacuum apparatus, direct paraffin-embedding, sectioning, dry-mounting, deparaffination in warm ether, treatment with silver nitrate, water, alcohol and water again. The essential point is control of the activity of the section by means of a reproducible setting under a G.M. tube at every step of the treatment. By this means it could be established that the treatment left the bromine *in loco*.

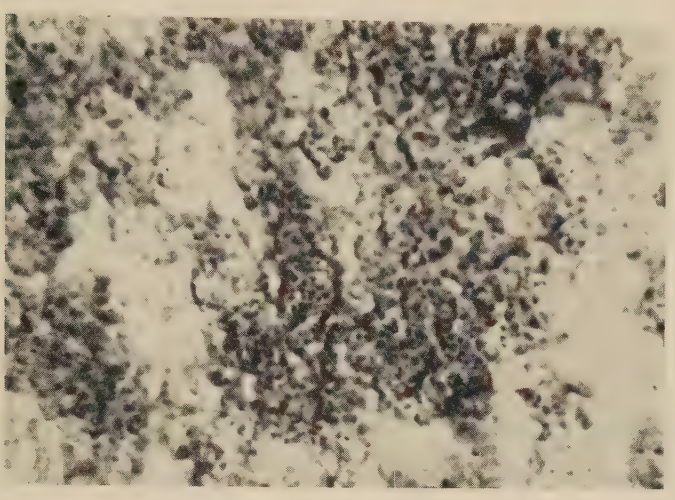

Fig. 3. Radioautogram from $Br^{82}$ over cells of the inner layer of the cortex of cerebellum. — ×1 500.

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# BRIEF NOTES

## UNFERTILIZED RABBIT OVA IN $S^{35}$-AUTORADIOGRAPHY

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The aim of the present work is to determine whether radioactive sulfate enters into the egg cell or not.

*Experimental.* Twentyfive anoestric female rabbits were given 1 mC $S^{35}$ as $Na_2SO_4$ per kg of body weight with about 0.1 mg carrier in 1 ml solution intravenously. After various time intervals (2, 4, 8, 16 and 24 hours, 2, 5, 10 and 16 days) the animals were killed. The ovaries were removed, fixed in methanol, passed through iso-propanol (3 hours), xylene (3 hours) and embedded in paraffin. They were cut in 10 $\mu$ thick sections, which were mounted on methacrylate slides, deparaffinized in xylene and ethanol and pressed against Gevaert Dentus Rapid film during 23–57 days for autoradiography. The film was developed for 30 minutes at +18.3° C. in Kodak DK 20 with KBr added to 0.2 per cent. The sections were stained according to Ladewig. The resolution obtained was 15–20 $\mu$, thus permitting the study of the egg cells.

*Results.* In all rabbits only small amounts of $S^{35}$ were incorporated by the egg cells from primordial, growth, and from Graafian follicles. Grain countings in the autoradiographic images revealed about 8 grains per 100 $\mu^2$ in areas corresponding to the egg cell, and about 17 grains per 100 $\mu^2$ in areas corresponding to the granulosa tissue (the values corrected for an exposure time of 30 days). The low uptake of $S^{35}$ in the egg cell is illustrated in Fig. 1.

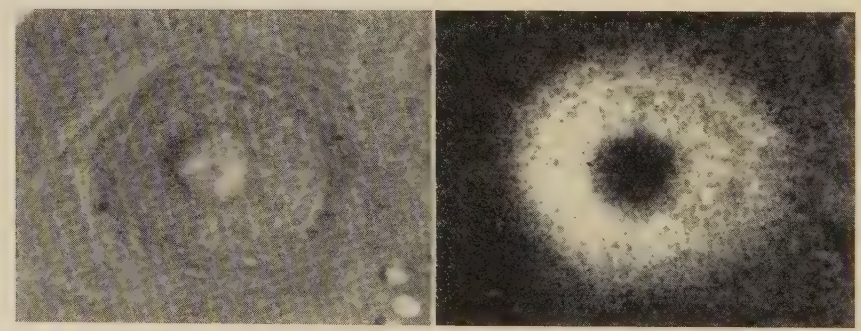

Fig. 1. To the left a growth follicle of a rabbit ovary (Ladewig stain). To the right the corresponding autoradiograph, showing the distribution of $S^{35}$. Light areas indicate $S^{35}$ activity. The granulosa tissue has a high uptake of $S^{35}$ but the ovum contains only small amounts. Injection of labeled sulfate 8 hours before death. Magnification 60×.

*Discussion.* It is surprising that an important element like sulfur is not incorporated into the egg cell. Of interest is the fact that the sulfur in the sulfate ion is not utilized in the synthesis of amino acids in the animal organism as shown by Tarver and Smith (4) but appears to enter rapidly into certain esterified polysaccharides (1, 3).

It is also interesting to compare the low incorporation of labeled sulfate in the unfertilized rabbit ovum with the low uptake of labeled phosphate, reported earlier (2).

*Summary.* The unfertilized rabbit ovum takes up only small amounts of labeled sulfate in its cytoplasm, as demonstrated by autoradiography.

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# ISOTOPE UPTAKE OF INDIVIDUAL CELLS

## TECHNIQUE FOR STAINED AUTORADIOGRAPHS AND MICROPHOTOGRAPHS FOR GRAIN COUNTING[1]

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Received June 2, 1952

In order to investigate the uptake of radioactive isotopes in bone marrow cultures it was essential to devise a method for determining the uptake and localisation in individual cells. The technical difficulties of localising a counter over a particular cell are great, and usually the amount of radioactivity per cell is too small for direct detection with such counters. The use of autoradiography eliminates these difficulties.

The best resolution is given by the stripping film technique (1, 3), but in order to eliminate as far as possible back scatter from the slide and lateral radiation from neighbouring cells it is important that the distance in between the cells should be at least twice the diameter of the cells. Bone marrow or blood cell suspensions cultured *in vitro* (2) yield smear preparations which fulfil these requirements. The morphology of the individual cells is well preserved, they are in a monocellular layer, and are suitably far apart.

For the work planned it was essential to be able to perform accurate differential counts on the autoradiographs, and it was therefore important to be able to stain the cells with one of the Romanowsky stains. Phase contrast examination, though giving valuable information about cytological details, will not allow differentiation of all cell types of the bone marrow with certainty.

Staining of the smears before coating them with the stripping film is contra-indicated. Not only is there a possiblility of removal of isotope from the cells during the staining process, but also the stain might produce artefacts in the photographic emulsion during the time of exposure, and finally the development and fixation might alter the staining very adversely. It has been found, however, that if the autoradiographs are carefully washed in running cold water for 4–6 hours after processing, and the optimal pH (6.8) is maintained by using buffered distilled water, then one hours staining with a 1 : 12 dilution of a Leishman-Giemsa mixture (1 part Leishman solution and 3 part Giemsa solution) gives as good staining as any ordinary smear.

The procedure is:— 1. Make smears on slides, fix smears for 1–2 mins in 95 per cent alcohol. 2. Coat smears with stripping film as described by Pelc (1, 3). 3. After exposure develop 4 mins. in Kodak D. 19b, fix for 15–18 minutes in half strength fixing solution. 4. Wash 2–4 hours in running cold tap water. 5. Stand for 2 hours

[1] This work has been done as part of an investigation covered by a grant from the British Empire Cancer Campaign.

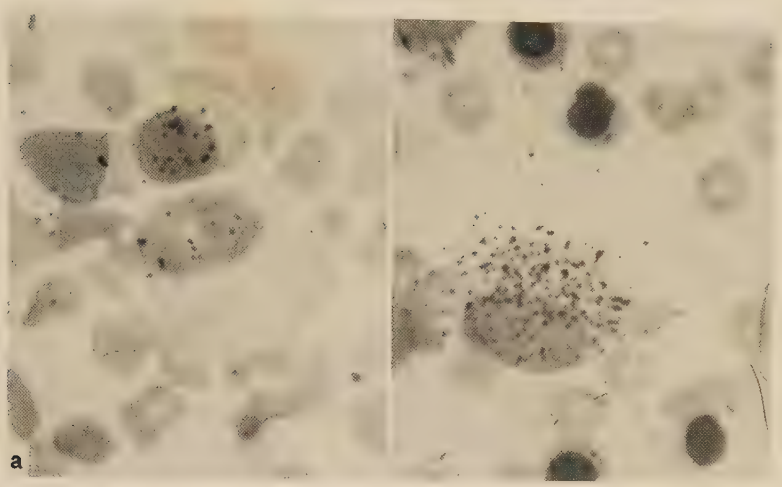


Fig 1. Fig. 2.

Fig. 1. Intracellular localisation of $P^{32}$. Bone marrow cells cultured 24 hrs in 1 μc $P^{32}$/ml medium. Stained autoradiograph, ×1000.
Fig. 2. Intracellular localisation of $S^{35}$. Bone marrow cells cultured 24 hrs in 5 μc $S^{35}$/ml medium. Note that nucleated red cells do not take up the isotope (sulphate form). Stained autoradiograph, ×1000.

in cold buffered (pH 6.8) distilled water. 6. Dry in air. 7. Stain with 1 : 12 Leishman Giemsa for 1 hour. 8. Wash off stain for 1 minute with buffered distilled water. 9. Dry autoradiographs in air.

If the cell suspensions are cultured in a medium containing isotopes it is important to ensure that, when the smears are made, as much isotope as possible should be removed from the suspending fluid. This can be achieved by several washings of the cells. Even after repeated washings some isotope will remain in solution and a brief washing of the slides with buffered distilled water after the smears have been fixed (before coating them with the stripping film) will reduce the background considerably.

The theory of autoradiography with particular reference to exposure time and isotope concentration is excellently dealt with in detail by Pelc (4). It is not worth while to expose longer than two half lives of the isotope in question, and in most cases considerably less time will be needed. The exposure will depend on the amount of isotope taken up by the individual cells. For example, 14 days' exposure of smears from cultures which have been grown for 6 hours in 5.0 μc/ml $S^{35}$ gives good autoradiographs in which the grains can be counted easily, the same exposure of the 24 hour cultures give too strong autoradiographs. For comparisons in groups of experiments certain standard exposure times may be needed. In this laboratory the standard exposure times for the culture smears are: $S^{35}$: 14 days, $P^{32}$: 21 days, Au $^{198}$: 3–4 days, $Sr^{90}$: 16–18 days.

The microphotographs Figs. 1 and 2 are from autoradiographs of bone marrow cultures grown with $P^{32}$ and $S^{35}$ respectively in the medium. They were made by the stripping film technique and stained afterwards as described above. The black granules clearly indicate the nuclear localisation in the case of $P^{32}$ and the mainly cytoplasmic localisation in the case of $S^{35}$. Knowing the grain yield per electron of a particular isotope, the exposure time (percentage decay of isotope in the cell), and the conditions for back scatter, an approximate but fair idea can be gained as to the actual content of isotope in the cell. The presence of some 50–60 $P^{32}$ or $S^{35}$ atoms per cell should be detected in this way, and even if a 100 per cent error range upwards, is assumed, the technique is a very sensitive one.

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# THE STRUCTURE OF THE EPITHELIAL SURFACE IN THE OLFACTORY REGION

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Within the olfactory area in the nose there are generally described three different types of cellular elements, basal, sustentacular and sensory cells. Only the two latter of these elements are said to reach the epithelial surface. The basal cells form a simple layer at the border to the underlying connective tissue.

The sustentacular and sensory elements are both said to extend throughout the whole epithelial layer. The former of these two form a supporting structure and according to many authors they end at the surface as small cuticular plates. The sustentacular cells are connected with each other leaving small openings through which the uppermost parts of the olfactory or sensory cells protrude. There is a rather general acceptance of the arrangement of the olfactory region so far described, but the opinions differ as to the structure of the surface.

Some authors describe and present pictures where the sustentacular cells are lacking any special kind of surface organelles (8), others describe single flagellar structures on the surface of these cells (10), whereas a third group of authors discuss the existence of a kind of striated border (5, 7). Their possible origin from broken off cilia is hereby suggested (5).

There are also diverse opinions as to the structure of the sensory cells. Most authors describe a peripheral vesicular ending of these cells.

In most text- and handbooks the occurrence of such olfactory vesicles is accepted, although a few authors regard them as artefacts (3). These olfactory vesicles are said to have a few cilia with basal bodies on their free surface 5–6 in man (9), 5–8 in pig (1), 10–14 in rabbit (6).

The olfactory cilia are generally described as 1–2 μ long processes about 0,1 μ in diameter and they end in blunt, sometimes thickened tips. They have a basal body, but no intracellular processes or rootlets. Kolmer therefore regards them as modified flagellae.

The calibre of these hairs is however of dimensions below the resolving power of the common light microscope and the description will consequently be inexact.

The electron microscope permits a closer study of these elements and in a large series of specimens from man and animals we have devoted them our interest. A fuller report of these studies will be published elsewhere (Bloom, Engström and Bloom).

Both with light and electron microscopy we have found the epithelial surface of the olfactory region to contain a very large number of thin, fingerlike, thinwalled processes. They have a length of about 2 μ and a diameter just below 0.1 μ. They are extremely thinwalled and show a fine granular surface in osmic acid fixed pre-

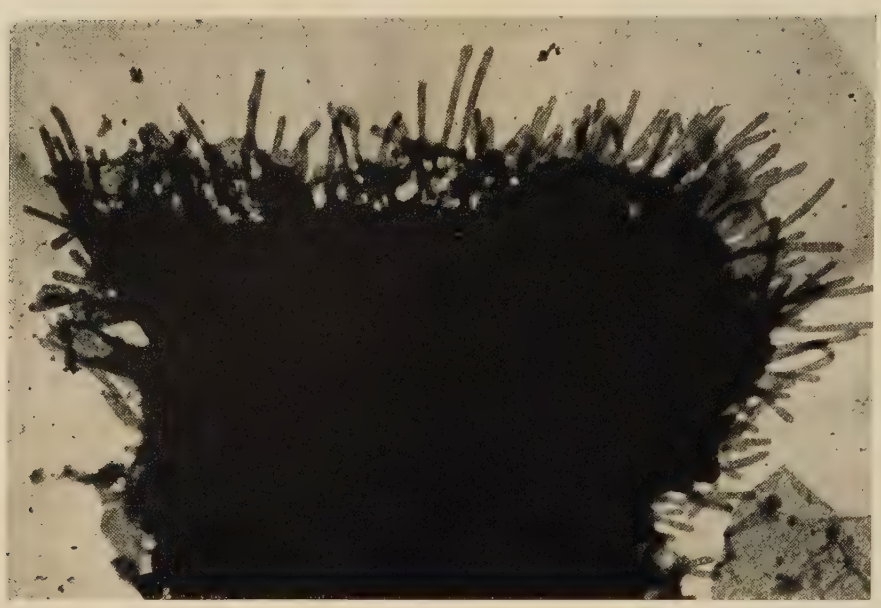

Fig. 1.

Fig. 2.

parations. They are very numerous on each studied cell, and in several cases we have calculated them to more than 1000 per cell. They have no distinct basal bodies, no intracellular rootlets and no resemblance to vibratile cilia as described by Engström (4) and others.

On the other hand, they have many similarities to the striated bord as found in the intestinal tract.

By means of the described processes the olfactory region acquires an enormous enlargement of its free surface, which must be of the utmost importance for its physiology.

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Fig. 1. Surface of an epithelial cell from the human olfactory region (female, 61 yrs.). A great number of thin, fingerlike processes with a length of 1,5–2 microns are seen. They are of a rather uniform length and width and end in blunt tips. A few of the latter even seem to be somewhat thickened. Osmic acid fix. Electron micrograph. Shadow cast with Pt + Pd. Magnification: 9000 ×.
Fig. 2. A higher magnification of the thin processes from the same case as above. Magnification 37000 ×.

# INTERRELATIONS BETWEEN NUCLEIC ACIDS AND GROWTH OF THE HUMAN PLACENTA

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The growing cell is characterized by a high concentration of nucleic acids (16, 13, 15, 11). Caspersson and Schultz (5) and Caspersson (4), by applying the ultra-violet microscopic technique, have demonstrated the dominant rôle played by pentose nucleic acid (P.N.A.) in the concentrative accumulation of nucleic acids in rapidly dividing tissues. The same results have been obtained by Brachet (2, 3), using histochemical methods.

On the other hand, the decreasing growth activity during cell maturation can be correlated to a continuous decrease of the concentration of P.N.A. This has been shown for blood cell-forming tissue by Thorell (22, 23) and for dentine cells by Thorell and Wilton (25).

Much interest has also been devoted to the biochemical study of the nucleic acid content in the tissues during embryonic development. After the introduction of the methods of Schneider (20) and Schmidt and Thannhauser (19), some recent contributions to these questions have been offered by Novikoff and Potter (18), Davidson, Leslie, Smellie and Thomson (9), Davidson and Leslie (8), and others.

However, no investigations concerning the nucleic acid content of the developing placenta have been made previously. This paper is a preliminary report of some analyses of the nucleic acid content in the human placenta during different stages of pregnancy, carried out as part of a study on the growth and differentiation processes of the placental tissue.

Placentas were obtained from provoked abortions on healthy women on social-medical indications according to Swedish law, and at parturitions. A detailed account of the methods for determination of nucleic acids will be published elsewhere.

*Results.* As a screening test 20 placentas were investigated according to the Schmidt and Thannhauser (19) procedure. The pentose nucleic acid phosphorus desoxypentose nucleic acid phosphorus (P.N.A.P./D.N.A.P.) quotients have been calculated, and the results are summarized in Fig. 1. The present writer has found that the Schmidt and Thannhauser procedure is not directly applicable to quantitative determinations of nucleic acids in these tissues. The main error, however, seems to be systematic (to be published), and changes in the P.N.A.P./D.N.A.P. quotient will mostly be determined by quantitative changes in the nucleic acids. Thus, the quotients express the relative changes during development.

Four placentas were investigated by the methods of Hammarsten (12) and

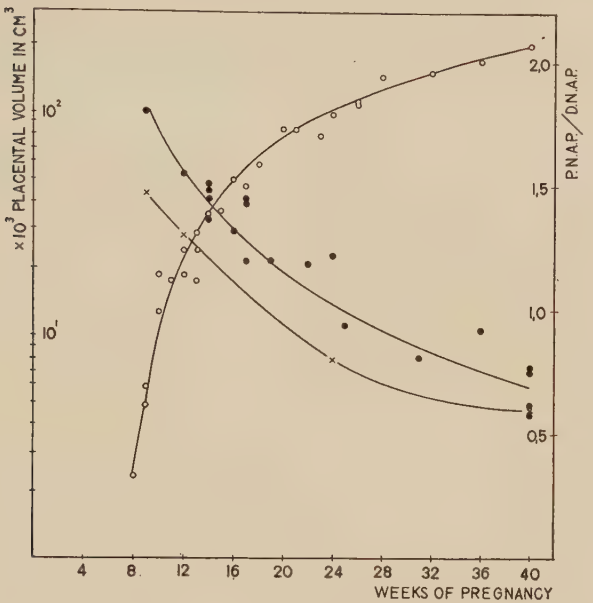


Fig. 1. Relationships between the P.N.A.P./D.N.A.P. quotients and volume of placenta during development.

×——× P.N.A.P./D.N.A.P. obtained by the extraction method of Hammarsten (12).
●——● P.N.A.P./D.N.A.P. determinations by the method of Schmidt and Thannhauser (20).
○——○ log volume of placenta.

Schneider (20). The results are given in Table I. The concentration of nucleic acids decreases with the aging of the placenta. This decrease is brought about by the reduced P.N.A.P. content per unit dry weight. The concentration of D.N.A.P. increases slightly. Accordingly, the P.N.A.P./D.N.A.P. ratio shows considerable changes.

TABLE I

| Method | Hammarsten | | | Schneider | | |
|---|---|---|---|---|---|---|
| Weeks of pregnancy | P.N.A.P. | D.N.A.P. | P.N.A.P./D.N.A.P. | P.N.A.P. | D.N.A.P. | P.N.A.P./D.N.A.P. |
| 9 | 2 665 | 1 815 | 1.47 | 2 500 | 1 750 | 1.43 |
| 12 | 2 315 | 1 790 | 1.30 | 2 495 | 1 765 | 1.41 |
| 24 | 1 805 | 2 270 | 0.80 | 1 760 | 2 245 | 0.78 |
| 40 | 1 210 | 2 040 | 0.59 | 1 235 | 2 060 | 0.60 |

P.N.A.P.: Pentose nucleic acid phosphorus.
D.N.A.P.: Desoxypentose nucleic acid phosphorus.
The figures are given as μg phosphorus/gram dry tissue powder.

TABLE II

| Part of placenta | P.N.A.P. | D.N.A.P. | P.N.A.P./D.N.A.P. |
|---|---|---|---|
| Foetal plac. | 1 825 | 2 345 | 0.78 |
| Decidua | 1 925 | 1 480 | 1.30 |
| Foet. membr. | 1 280 | 895 | 1.43 |

P.N.A.P.: Pentose nucleic acid phosphorus.
D.N.A.P.: Desoxypentose nucleic acid phosphorus.
The figures given as μg phosphorus/gram dry tissue powder.

In a 36-week old placenta the nucleic acids and the quotients in the decidua, the foetal placenta and the foetal membranes were determined (Table II). The differences are noteworthy and will be further studied.

*Discussion.* Stieve (21) made an investigation on the growth of the human placenta from a morphologic point of view. He measured the thickness and the diameter of placentas at different stages of development. On the basis of these figures the present writer has calculated the placental volumes, assuming that this organ may be regarded as a spherical segment. The volume is taken as a physical measure of the growth of the placenta. Fig. 1 shows the curves for the P.N.A.P./D.N.A.P. quotients and the placental volume increment.

On the presumption that the D.N.A.P. concentration in the single cell undergoes but small changes (1, 26, 27, 17, 6, 7, 24, 10) the P.N.A.P./D.N.A.P. quotient is a relative expression for the average P.N.A.P. concentration of the cell population. The figure indicates the high cellular concentration of P.N.A.P. during the period of logarithmic growth. During this stage of growth the P.N.A.P./D.N.A.P. curve also shows the greatest slope.

From the data it may be justifiably presumed that the period of rapid growth is preceded by a period of accumulation of P.N.A. in the cell. This is in agreement with the results obtained by Thorell (23) by studies on blood cell-forming tissue, by Malmgren and Hedén (14) on bacteria, and others.

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# THE BEHAVIOR OF CHICK HEART FIBROBLASTS IN SOME PROTEIN-FREE MEDIA

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Attempts have been made during recent years to maintain and grow animal tissue cells in entirely synthetic media based on the assumption that survival and growth of the cells can take place without the presence of proteins in the medium (12, 13, 14, 15). In these investigations no convincing evidence of growth was ever produced while survival for limited periods of time was obtained.

It has been shown by other methods, however, that tissue cells *in vitro* cannot survive and grow unless a suitable mixture of low molecular weight compounds is part of the medium (1, 2, 8). It was therefore thought worth while to investigate whether any mitotic divisions were taking place in a few protein-free media at our disposal (White's synthetic medium, serum ultrafiltrates from horse and chicken and a dialysate from embryonic chicken extract).

*Technical procedure:* Fresh explants were taken from hearts of chick embryos of 9–10 days of incubation and placed on a sheet of perforated cellophane on the bottom of a Carrel flask (see 6); 4–5 explants in each flask. The medium (approx. 1 cc.) was renewed every 2–3 days. The pH was kept approximately within the limits of 7.4–7.8 judged by the color of phenol red which was included in all the media employed. During the usual cultivation period of two to three weeks zones of migrating cells were formed around the explants on the cellophane surface.

The estimation of the mitotic frequency was made by counting the mitoses and the resting nuclei in randomly selected areas of the migration zones after fixation in Ringer Formol and staining by Delafield Hematoxylin.

The synthetic medium was prepared as described by White (14, 15) and the serum ultrafiltrates were obtained from "Microbiol. Assoc.", Washington, D.C. USA. The embryo juice dialysate was prepared by dialysing the undiluted, crude extract toward an equal volume of Earle's saline solution for 24 hours in a refrigerator. The embryo juice was made by forcing the embryos through a 50 cc. syringe and the fluid produced thereby was separated from the tissue pieces by centrifugation.

In the first 3 series of experiments the cell colonies were maintained for two to three weeks in White's medium before they were given the experimental medium; in the fourth series, they were maintained in embryo juice dialysate the entire time. Within each experiment about 1000 nuclei were counted in each of 5 different cell colonies. Table I shows the kind of results which were obtained.

It is quite evident from the results of these experiments that no growth was taking

TABLE I

| Prelim. med. Time in days | Exp. med. Time in hours | Number of nuclei | Number of mitosis | Number of mitosis per 1 000 cells |
|---|---|---|---|---|
| 1. White's Med. | White's Med. | | | |
| 12 | 24 | 5 448 | 2 | 0.4 |
| 14 | 24 | 1 105 | 1 | 0.9 |
| 19 | 24 | 5 081 | 0 | 0 |
| 20 | 40 | 5 810 | 2 | 0.3 |
| 29 | 50 | 5 308 | 0 | 0 |
| 2. White's Med. | Chick serum ultrafiltrate | | | |
| 22 | 24 | 5 051 | 10 | 2 |
| 25 | 48 | 5 411 | 2 | 0.4 |
| 25 | 48 | 5 243 | 1 | 0.2 |
| 15 | 72 | 5 754 | 9 | 1.6 |
| 3. White's Med. | Horse serum ultrafiltrate | | | |
| 14 | 24 | 5 221 | 15 | 2.9 |
| 4. Embryo juice dialysate | Embryo juice dialysate | | | |
| 10 | 24 | 5 075 | 2 | 0.4 |
| 13 | 24 | 5 349 | 5 | 1.0 |
| 14 | 24 | 5 254 | 10 | 2.1 |
| 12 | 48 | 5 087 | 6 | 1.2 |

TABLE II

Experimental medium: a mixture of horse serum (40 per cent), embryo juice (20 per cent) and Earle's saline solution (40 per cent)

| Time in embryo juice dialysate | Time in exp. medium | Number of nuclei | Number of mitosis | Number of mitosis per 1 000 cells |
|---|---|---|---|---|
| 12 days | 24 hours | 5 614 | 37 | 6.6 |
| 17 „ | 24 „ | 5 191 | 53 | 10.2 |
| Time in horse serum etc. | | | | |
| 7 days | 24 hours | 2 738 | 25 | 9.1 |

place in the migration zones due to constituents of the media given to the cells; it appeared, however, that they are able to survive for some time under these circumstances.

By the same procedure the mitotic frequency in a medium known to support growth of tissue cells was determined for comparison. In one series of experiments, the cell colonies were kept in embryo juice dialysate for 12–17 days before the experimental medium was used; in the other series they were maintained in the experimental medium continuously. The results of these experiments are given in Table II.

The mitotic frequency found in these experiments is no doubt significantly higher than the values found for the protein-free media but surprisingly low as compared with the figures found in the literature. The mitotic frequency in rapidly growing fibroblast cultures in a medium consisting of blood plasma and embryo juice is known to be of a magnitude of 20–30 mitoses per 1000 nuclei (7).

Several experiments were performed to determine the length of time the cell colonies could survive in protein-free media.

Three series of cultures were kept in White's medium for 45 -52 days. During this whole period cells were migrating from the explants out on the cellophane surface where they degenerated and died after a few weeks. Degenerating cells were in fact nearly always found in the periphery of the migration zones. Toward the end of the cultivation periods the explants were in an advanced state of disintegration and the migration zones were very much reduced in size; this is essentially a confirmation of White's results.

The establishment and maintenance of cell colonies with migration zones in the serum ultrafiltrates gave rather discouraging results. These migration zones were much less developed than was observed in White's medium. Furthermore, the cell colonies could not be kept alive for more than about 20 days in this medium.

In spite of the fact that a more extensive migration of cells takes place in embryo juice dialysate than in White's medium, the migration zones and explants were also found to be very much reduced in size after prolonged cultivation. One series of cultures was carried through 38 days of cultivation in the usual way, and it was evident by the end of that time that the cells would never cover the entire bottom of the flasks.

It was of interest to notice that in the protein-free media employed here the embryonic heart fibroblasts showed hardly any sign of the formation of fat droplets in their cytoplasm, which otherwise is so characteristic of many cell types (including heart fibroblasts) in the presence of blood plasma proteins in the medium.

It has been shown by Jacoby and Darke (10) that chick macrophages do not survive and grow in White's medium and the evidence produced in the present paper further supports the view that White's medium alone may not be a suitable basis for growth (see also 4, 11).

The effect of embryo juice dialysate alone on tissue cells *in vitro* was studied by Wright (16) who claimed that he observed an increase in the mitotic frequency, a finding which has not been confirmed by the present investigation.

In view of the fact that earlier investigators (1, 2, 3, 8, 9) have shown by various methods that serum ultrafiltrates and embryo juice dialysates contain substances of importance for cellular survival and growth it seems of interest that in the present investigation no sign of growth was found in these protein-free media.

This would suggest that much more extensive work along these lines is necessary before the question whether or not protein types as such in the culture medium are essential for the survival and growth of animal tissue cells.

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